

Environment. Industrial Wastes Branch.

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Ministry of the ENVIRONMENT

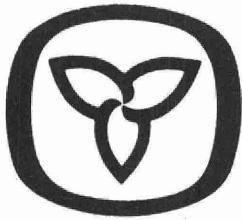
Acceptability Criteria
and
Testing Procedures
for
Oil Spill Treating Agents
1972

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Environment Ontario

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MINISTRY OF THE ENVIRONMENT

ACCEPTABILITY CRITERIA

AND

TESTING PROCEDURES

FOR

OIL SPILL TREATING AGENTS



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Special Projects Section - Chemical

INDUSTRIAL WASTES BRANCH

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INTRODUCTION

Spillages of oil associated with transportation, manufacture, drilling or other oil handling activities present a danger to aquatic life as well as constituting a nuisance and an aesthetically unpleasant condition.

Obviously, spill prevention is the most desirable approach to safeguard against damage to the environment, but spills will continue to occur through negligence, accidents and other unforeseen circumstances. Impoundment of spills when they occur is the next order of priority with physical removal of the oil next in order of importance. Under certain conditions, however, the foregoing procedures may be impractical, inadequate or unsafe. In these cases, oil spill treating agents (OSTA) may be used in accordance with guidelines provided by the Ministry of the Environment and appended to this report (Appendix V).

This report deals with the criteria of acceptability for chemical OSTA to be used where containment and clean-up procedures prove impractical or where secondary clean-up following such procedures becomes necessary.

Factors such as effectiveness and potential adverse effects on the environment must be assessed before chemical treating agents are approved for use on oil spills.

An approved list of oil spill treating agents will therefore be prepared based on the criteria contained in this report.

CRITERIA FOR ACCEPTABILITY OF OIL SPILL TREATING AGENTS

Toxicity

The maximum amount of the oil spill treating agent permitted per 100 square feet of surface area shall not exceed an amount

which would give rise to a concentration, in the top one foot of water, equivalent to the 96-hour TL_m , as measured by methods approved by the Ministry of the Environment. The maximum amount of oil spill treating agent that may be used in a particular situation will be calculated by multiplying the surface area to be treated in 100 square feet by the number of litres per 100 cubic feet (2831.6 litres) and by the 96-hour TL_m in milligrams per litre.

Notwithstanding the foregoing, oil spill treating agents having an average level of toxicity, as determined by the required toxicity methods (oil spill treating agent and oil spill treating agent plus oil), more toxic than 100 mgm/litre (96-hour TL_m less than 100 mgm/litre) shall not be approved.

Degradability

Acute toxicity shall not be measurable for a 96-hour period in an aqueous solution of the oil spill treating agent after 20 days of aging at 15°C and conditions of aeration using discrete bubbles from a three mm ID glass tube. The time of aging for the solution is the time at the end of the 96-hour period. The initial concentration of the test solution shall be 10 times the 96-hour TL_m in mg/litre of the species of fish used in the test.

Performance Effectiveness

If more than 10 percent of the treated oil sinks, the agent shall be considered as a sinking agent and as such is subject to the restrictions pertinent to these agents.

A variety of test procedures have been developed and adopted by various agencies to evaluate the effectiveness of OSTA's. None of these has been found to give reproducible results or results

that can be precisely correlated to the performance of dispersants under field conditions.

As a means of determining whether the particular oil spill treating agent is effective for the function claimed, manufacturers and suppliers must submit data on performance effectiveness together with a detailed description of the test procedures used. For the benefit of applicants, two test procedures applicable to dispersants are appended to this report. These are the SET (Simulated Environmental Tank) procedure and a bench test developed by Ministry of the Environment Research Branch Laboratories.

Ideally, performance effectiveness testing should be based on one or more of the standard reference oils used in the bioassay procedures. Namely, No. 6 fuel oil, Leduc crude oil or Lloydminster crude oil.

Metals

Toxic metals may not be a constituent of the oil spill treating agent. Mercury and cadmium should be virtually absent from the oil spill treating agent to the limit of current analytical techniques.

Chlorinated Hydrocarbons

Chlorinated hydrocarbons, including but not limited to, solvents, poly-chlorinated biphenyls and terphenyls, hexachlorobenzene, pesticides and herbicides, may not be a constituent of the oil spill treating agent. Total identifiable chlorinated hydrocarbons may not be present in excess of 0.05 milligrams/litre of the oil spill treating agent.

Sinking Agents

Sinking agents will not be approved for use in the surface waters of Ontario. However, as a means of dealing with

oil spills in confined situations such as in sewage treatment works or industrial waste treatment plants, sinking agents may be approved on an individual basis. Manufacturers or suppliers of sinking agents may therefore wish to submit toxicity and other data on their product so that it may be approved for limited use as outlined above.

Use of Dispersants on Light and Heavy Oils

From the point of view of use, the treatment of spills of #2 fuel oil and less viscous distillate fuels with dispersants is not warranted. If not treated, distillate fuels will disperse rapidly by natural processes of weathering. Also, dispersing agents tend to enhance the toxicity of distillate fuels.

Because of the difficulties encountered in mixing dispersants with heavy residual oils such as #6 fuel oil, the use of dispersants on spills of such oils is not recommended, particularly during cold weather.

OSTA Other Than Dispersants, Collecting Agents and Sinking Agents

Chemicals have been developed for aiding combustion of oil, gelling oil, enhancing the biodegradability of oil, etc., but in most cases, their applicability for use on oil spills is more limited. These chemicals shall not be used under any circumstances without prior consultation with staff of the Industrial Wastes Branch of the Minister of the Environment.

PROCEDURES FOR OBTAINING APPROVAL OF OIL SPILL TREATING AGENTS

The information required by the Ministry of the Environment in order to place a specific oil spill treating agent on the approved list is itemized in the following form which should be filled out by the applicant:

ONTARIO MINISTRY OF THE ENVIRONMENT
WATER MANAGEMENT
INDUSTRIAL WASTES BRANCH
135 ST. CLAIR AVENUE WEST
TORONTO

APPLICATION FOR APPROVAL OF OIL SPILL TREATING AGENT

Date _____

1. PRODUCT TRADE NAME _____

Name of Manufacturer _____

Address _____

Distributor in Ontario _____

Address _____

Technical Representative _____

Address _____

Telephone: Area Code _____ Number _____ Ext. _____

2. USE CLASSIFICATION OF AGENT:

Dispersant

Collecting Agent

Sinking Agent

Other _____

3. MATERIAL CLASSIFICATION AND ANALYTICAL DATA:

Chemical name and percentage of each component (when other than of natural origin). This information will be treated as confidential by the Ministry and its agents.

Concentrations in mg/l of:

lead _____ Mercury _____

cadmium _____ Total chlorinated
hydrocarbons _____

Solubility at 15°C in standard fresh water _____

Color (visual) _____ Flash Point _____ °F

Freezing Point _____ °F Specific _____ @ _____ °C
Gravity

Chemical Oxygen Demand mg/l _____

pH _____

4. HAZARDS TO OPERATORS

Inhalation (acute LC₅₀ to _____) _____

Skin irritation or sensitivity concentration _____

Eye irritation _____

Sensory Threshold Properties _____

Hazardous Gases Produced on Combustion _____

Chronic Hazards _____

5. POTENTIAL EFFECTS ON PUBLIC SURFACE WATER SUPPLIES

Threshold Odour Number (see Appendix VI) _____

Phenolic Equivalent ppb (see Appendix VII) _____

Acute Oral Toxicity LD₅₀ mg/kg _____

6. TOXICITY TO AQUATIC LIFE

Acute toxicity of agent to pimephales promelas (fathead
minnow) in standard fresh water.

TL_m 96 hours at 25°C ± 1°C = _____ mg/l

Acute toxicity to other aquatic organism(s)

<u>Species</u>	<u>Type of Water</u>	<u>96-hr TL_m</u>
----------------	----------------------	-----------------------------

Highly variable
specification

Acute toxicity of 1:5 mixtures of OSTA and Leduc crude oil, Lloydminster crude oil and No. 6 fuel oil in standard fresh water to Pimephales promelas.

OSTA + #6 fuel oil - 1:5 TL_m 96-hr @ 25°C ± 1°C = ____ mg/l

OSTA + Lloydminster - 1:5 TL_m 96-hr @ 25°C ± 1°C = ____ mg/l

OSTA + Leduc - 1:5 TL_m 96-hr @ 25°C ± 1°C = ____ mg/l

Average 96-hr TL_m @ 25°C = ____ mg/l

7. DEGRADATION

ACUTE TOXICITY to the selected species shall not be measurable during a 96-hour exposure period in an aqueous solution containing a concentration of OSTA 10 times the initial 96-hour TL_m as measured after 20 days of aging at 15°C. (See bio-assay procedure, Appendix III)

PRODUCT DEGRADATION by quantitative analysis of the product in a solution of standard fresh water over a period of 20 days. (Attach report)

BOD (Method _____): 5-day _____ Ultimate _____ K _____

8. ANALYTICAL PROCEDURES (CHEMICAL)

Recommended analytical method to identify product.
(Attach procedure) _____

Recommended analytical method for determining as little as 5 mg/l of product in fresh water. (Attach procedure) _____

9. PERFORMANCE EFFECTIVENESS

Applicants must submit data on laboratory or pilot scale evaluation of the performance effectiveness of their products.

Detailed descriptions of the test procedures used should be submitted with this application.

For the information of applicants, two procedures which have been evaluated by the MOE Research Branch are appended (Appendix I and I-A).

10. OSTA SINKING CHARACTERISTICS

Sinking Test: (25 ml Leduc crude oil + 5 ml OSTA)
(25 ml Lloydminster crude oil + 5 ml OSTA)
(25 ml #6 fuel oil + 5 ml OSTA)

Millilitres of settled material in one hour:

Leduc crude oil	_____	ml
Lloydminster crude oil	_____	ml
No. 6 fuel oil	_____	ml

11. METHODS OF USE

Describe recommended procedures for use in open ocean, open bays and estuaries, docking areas and marinas, lakes, streams and rivers. (Attach report)

12. MISCELLANEOUS

Storage Depots of Product:

Address _____

Telephone: Area Code _____ Number _____

NOTES FOR APPLICANTS

Section 1

(i) Labelling Instructions:

Products should be labelled as follows:

(a) Brand name, trademark, etc., under which the product is sold.

- (b) Name and address of the manufacturer, importer or distributor.
- (c) Special handling, storage and safety precautions.
- (d) Flash point and freezing point of the product.
- (e) Recommended application procedures, dosages and conditions for use pertinent to water hardness and/or salinity, water temperature and types and ages of oils.
- (f) Maximum application rate per 100 ft² of oil slick to be treated.

Potential health hazards are of concern where these pose a threat to persons handling these materials or to the general public. The information requested on the health hazards may be subject to interpretation by the Ontario Ministry of Health. Recommendations as to safety precautions should, therefore, be clearly indicated on product labels and/or containers. Products which constitute a significant occupational health hazard will not be approved.

Section 2 - Self-explanatory

Section 3 - Material Classification and Analytical Data

Describe the product by generic type and percent composition of each type. Indicate the laboratory test procedures and instruments used in the heavy metal and other analyses where these differ from "Standard Methods for the Examination of Water and Wastewater" or the appended analytical procedures.

Hydrogen ion activity of the product as applied to the water surface shall be between 5.5 and 9.5. The limitations on

mercury, cadmium and chlorinated hydrocarbons are based on the known potential for these compounds to accumulate in fish and wildlife and present a human health hazard via dietary intake. Flash point is considered pertinent in that the use of OSTA's in the vicinity of oil fires may constitute an additional fire hazard where low flash point products are used. The Cleveland Open Cup flash point method is recommended (ASTM).

Section 4 - Hazards to Operators

Descriptions of test methods and test animals used to obtain the reported data must be submitted with the application. See also the notes pertinent to health hazards accompanying the instructions regarding labelling.

Section 5 - Potential Effects on Public Surface Water Supplies

Threshold odour number, phenolic equivalents and acute oral toxicity are significant where OSTA's may be used on surface waters which are sources of public water supply. While it is not anticipated that OSTA's will be used in the vicinity of municipal intakes, these data are necessary to assess the suitability of particular OSTA's in situations where there is some slight possibility that contamination of public water supplies may occur.

Section 6 - Hazard to Aquatic Life

Because of its almost universal distribution in Ontario, the fathead minnow (*pimephales promelas*) has been selected as the test animal for fresh water bioassays. Other fresh water organisms have also been proposed as alternates and these are discussed in the detailed bioassay procedure contained in this report.

Section 7 - Degradation

In accordance with the test procedure described later in this report, it is recommended that acute toxicity shall not be measurable for a 96-hour period in an aqueous solution after 20 days of aging at 15°C. The initial concentration of the OSTA to be used in the degradability tests shall be 10 times the average 96-hour TL_m of the species of fish used in the test. The time of aging for the solution is the time to the end of the final 96-hour toxicity test period. (See Appendix III)

Product degradation by chemical analysis over a 20-day period should be reported using the shake flash or similar techniques described by M. Mausner et al in Status of Biodegradability Testing of Nonionic Detergents. The Soap and Detergent Association Scientific and Technical Report No. 6, October 1969. A detailed laboratory report on this must accompany the application.

Biochemical oxygen demand data is to include the five-day, ultimate, and rate of reaction (K) values. Method of determination of the BOD is at the discretion of the applicant although the method used must be reported. The standard method for BOD is appended to this report (Appendix VIII).

Information on biochemical oxygen demand is sought for the purpose of regulating the oxygen load placed on inner harbour areas where insufficient oxygen may already be a limiting factor to the well-being of the environment. In addition, rate of biochemical oxygen demand is useful in an assessment of the biodegradability of the product especially when compared to toxicity decay results.

Section 8 - Analytical Procedures

Methods for qualitative and quantitative analyses of dispersing agents are described in this report. These methods or others of the applicant's choosing may be used; however, the actual procedure used must be reported along with the results.

Section 9 - Performance Effectiveness

The sinking test is to be performed and reported on for all OSTA.

Dispersants: In the case of dispersing agents, quality of performance is a function of the degree of dispersion of oil into the water and the time that the dispersed oil stays in emulsion in the water before returning to the surface. (If more than 10 percent of the oil sinks as opposed to being emulsified, the agent should be considered a sinking agent.) The action and quality of dispersion vary according to many conditions of the test, two of which are very significant -- type of oil and the physical mixing forces used. Because of these variables, tests can be designed for a given product that will provide a range of efficiencies of dispersion; consequently, to require the testing of a product for its performance effectiveness in all situations would be impractical. However, some data is needed among those products available to discriminate whether they are potentially useful as a dispersant or not. The Military Tank Test as modified by either the FWQA or this report (S.E.T. Procedure) and the laboratory procedure developed by the MOE Research Branch provide an opportunity to make such discriminations.

Based on tests performed during the preparation of this study, it is recommended that at least 15 percent of the #6 fuel oil remain in emulsion for six hours as determined using the S.E.T. test and as based on an average of five duplicate tests. If a product is excluded from the list because it does not meet this minimum condition of dispersion with #6 fuel oil, the manufacturer must convince the Ministry that the test is not applicable to his product and, in the course of his protest, produce quantitative evidence through alternative testing methods that the product does disperse oil.

Collecting and Sinking Agents: The manufacturer or supplier must submit data in performance effectiveness using a test method of his choosing.

Section 10 - OSTA Sinking Characteristics

Sinking of oil spills is considered to be unwanted and unwarrented in natural watercourses. Although sinking of oil is normally achieved with products specifically designed for this purpose, other OSTA may cause oil to sink. All OSTA must therefore be subjected to the screening test outlined in the first part of Appendix II. If more than 10 percent of the treated oil sinks, the OSTA will be considered a sinking agent for the purposes of this application.

Section 11 - Methods of Use

Self-explanatory.

Section 12 - Miscellaneous

Data to be used in the routine management of OSTA and in case of disaster when large quantities of a particular product may be needed.

As explained in other sections of these instructions, considerable latitude is allowed the applicant in the manner of testing. Consequently, it is necessary that all laboratory reports submitted with the application be detailed and complete, because in some instances interpretation of results will determine whether or not confirmatory testing is to be done.

References

- (1) Evaluating Oil Spill Cleanup Agents - Development of Testing Procedures and Criteria, California State Water Resources Control Board.
- (2) Proceedings of the Joint Conference on Prevention and Control of Oil Spills, API-FWPCA, December 1969, New York.
- (3) Chemical Treatment of Oil Slicks, FWPCA, March 1969.
- (4) Oil Dispersing Chemicals, FWPCA, March 1969.
- (5) Gelling Crude Oils to Reduce Marine Pollution From Tanker Oil Spills, EPA, January 1971.
- (6) Oil Spill Treating Agents Test Procedures, Status and Recommendations, API Publication #4053 by Battelle Northwest Institute, May 1970.

ACKNOWLEDGEMENTS

- (1) Bioassay procedures prepared by D. Wells, Biology Section.
- (2) Effectiveness testing procedure prepared and evaluated by A.Oda, Research Branch.

APPENDIX I

EFFECTIVENESS TESTING - S.E.T. PROCEDURE

Because there are a large number of unknown variables which can, under field conditions, influence the dispersing action of a chemical dispersant on an oil slick, it is difficult to simulate field conditions in the laboratory to measure the effectiveness and performance of chemical dispersants. However, before any chemicals can be applied to treat an oil slick, it is necessary to obtain relevant information whereby the usefulness of a particular chemical agent can be properly judged.

Principle of Method

This method was developed by the U.S. Navy to measure the oil-emulsifying capability of solvent bilge cleaning agents and was modified by FWPCA and the California State WRCB for measuring the performance effectiveness of chemical dispersants. It involves the use of a simulated environmental tank.

A sample of chemical dispersant is sprayed on an oil slick formed on the surface of the water in this tank. The water is recirculated continuously and samples are withdrawn at given intervals and analyzed to determine the amount of dispersion and the stability of the emulsion.

Materials for the S.E.T. Tank

One 45-gal drum epoxyl-coated
Recirculating pump
Powerstat
½-inch ID PVC pipe, fittings, etc.
Clamps and miscellaneous parts
Tygon Tubing - 3/16-inch ID
2 gal polyethylene bottle
25-ft. reinforced garden hose and nozzle

Laboratory Equipment Required

Spectrophotometer and its accessories
100-ml volumetric flasks
500-ml separatory funnels
250-ml separatory funnels (one)
glass funnel and filter paper
500-ml graduated cylinders and other laboratory glassware

Testing with a Reference Dispersant

- (1) Mix 2-ml of standard surfactant (IGEPAL CO 630, GAF Corporation) with 25-ml 2-methyl-2, 4-pentanediol (hexylene glycol). Then add this solution to 100 ml of test oil in a 250-ml separatory funnel and mix by agitating vigorously for 10 minutes. Then place on the surface of the water in the SET tank without the ring and hose with water immediately. A 10-minute sample should be collected and analyzed as indicated below to obtain the initial dispersion value.

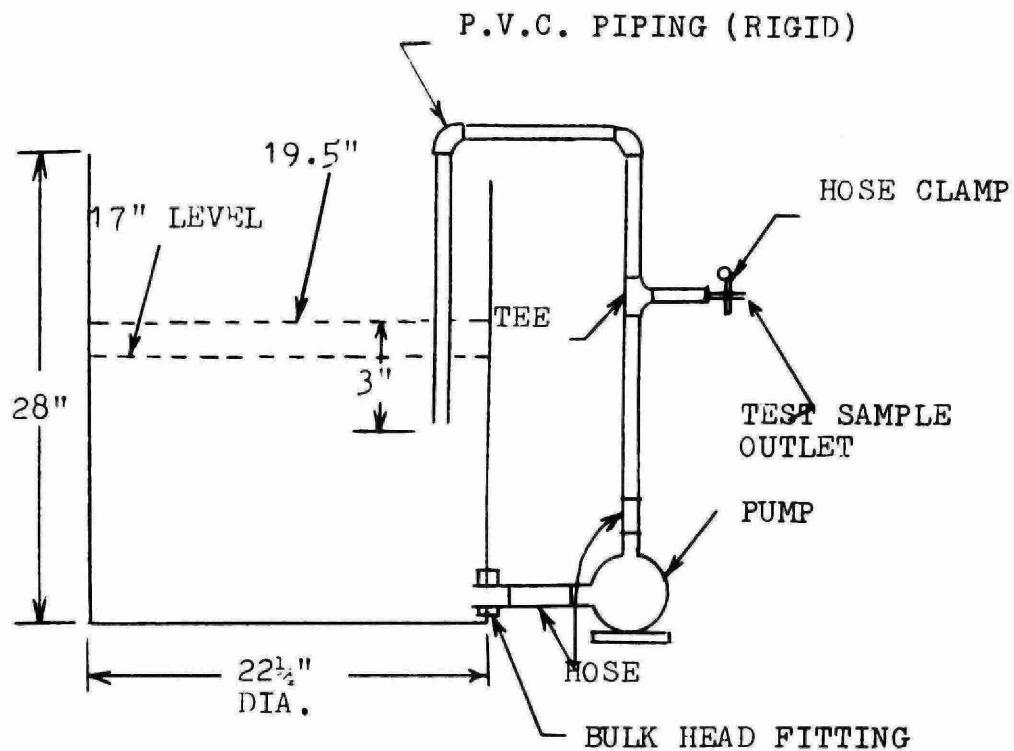


Figure 1 Set test apparatus. Standard 55 gallon steel drum with epoxy paint. Polyethylene centrifugal pump equal to Cole-Parmer No. 5790. Polyethylene tubing, 5/8" ID. Polyvinyl chloride (PVC) piping (rigid), $\frac{1}{2}$ " thin wall. Test sample outlet consisting of 3/16" ID Tygon tubing and a hose clamp. Total volume at 19.5" level, of approximately 130 liters.

Procedure

- (1) Fill the 45-gal drum to the 17-inch mark with the test water.
- (2) Place the floating plastic ring in the centre of the tank so that it extends 4.5 inches below the surface of the water. This can be done by fitting two flotation collars around the outside of the plastic cylinder. Each collar is constructed of 3/4-inch diameter tygon tubing connected at the ends with $\frac{1}{2}$ -inch PVC tubing. One collar is placed on the cylinder so that five inches extends below the surface of the water and the other placed above it so that the cylinder will remain in an upright position in the water. The floating plastic ring is held in place by means of strings tied to laboratory clamps which are secured to the rim of the tank.
- (3) With the recirculating pump off, pour carefully approximately 100 grams of the test oil into the centre of the "ring". The exact amount of oil should be determined by weighing the dispensing container before and after the addition of the oil.
- (4) Allow 5 minutes for the oil slick to stabilize and come to equilibrium with the water temperature.

(5) Add 30 grams of the dispersant by spraying in a fine stream through an aperture of 3 mm. contacting as much oil as possible. A small polyethylene laboratory wash bottle is suitable for this purpose. The dispersant must be applied in less than two minutes. Weigh the bottle before and after the application of the dispersant to obtain the weight of the dispersant.

(6) Wait 10 minutes to allow the dispersant to contact the oil inside the ring.

(7) Agitate the oil-chemical mixture using tap water from $\frac{1}{2}$ -inch inside diameter rubber or plastic hose with a 3/16-inch inside diameter orifice and water pressure of 50 psi. Initially, the water from the hose should be directed momentarily into the inner wall of the "ring" as the ring is being lifted out of the water to remove the adhering oil and chemical. Agitation of the surface of the water in the tank should be continued until the volume in the tank has reached 19.5 inch. In some cases, the high foaming characteristics of some dispersants make it difficult to see the 19.5-inch mark. This problem can be overcome by timing the interval required to reach the 19.5-inch mark prior to the test and duplicating this interval during this stage of the

test procedure.

(8) As soon as the hosing is completed, start the recirculation pump and continue throughout the test.

(9) Ten minutes after the termination of hosing, withdraw a 500-ml sample to determine the amount of initial dispersion. Before taking the actual sample, the sampling line should be cleared first by collecting a few hundred ml. in a beaker. After taking the sample, return the collected oil-water mixture to the tank.

(10) Immediately after collection, transfer 500-ml sample quantitatively to a separatory funnel and extract the dispersed oil with four 20-ml portions of chloroform. Filter the chloroform extract through anhydrous sodium sulphate and dilute to 100-ml.

(11) Determine the concentration of oil at suitable wavelengths with a spectrophotometer. (A standard calibration curve should be prepared by plotting absorbence vs. oil concentration for the oils used by selecting a suitable wavelength.)

(12) The general stability of the emulsion can be determined by analyzing samples withdrawn at 2 hours and 6 hours.

(13) Before the calculations are performed, corrections must

be made to the spectrophotometer readings. This provides for the sample blank of the dispersant and the natural detergent qualities of the test water. The sample blank for the chemical dispersant is determined by performing the above test with the dispersant in the absence of oil. The "oil blank" which refers to the natural detergent qualities of the test water is determined by performing the test with the oil alone in the absence of the dispersant.

Calculations

Calculate the percentage of oil dispersed as follows:

$$\% \text{ dispersion} = \frac{A}{B} \times C \times 100$$

where A = weight of oil in 500 ml sample

B = volume of sample (500 ml)

C = volume of SET tank

D = weight of oil used in the test

Reporting

Data collected should be reported as in the attached form.

**EFFECTIVENESS TESTING OF OIL SPILL CHEMICALS
USING MODIFIED S.E.T. METHOD**

Test No. Date By

Oil Sample Chemical

Supplier:
.....

Weight: Before gm
After gm
..... gm
Weight: Before gm
After gm
..... gm

Method of Application

Test Water from

Pump on Recirculation Rate litres/min.
off U.S. gpm

Observations

Oil Analysis and Percent Dispersion

<u>Time</u>	<u>Temperature</u>	<u>Reading</u>	<u>Amount</u>	<u>Corrected</u>	<u>% Dispersion</u>
Oil Blank °C
.....
.....
.....
.....
.....
.....

Comments

.....
.....
.....

APPENDIX IA

EFFECTIVENESS TESTING

ONTARIO MINISTRY OF THE ENVIRONMENT

RESEARCH BRANCH

LABORATORY PROCEDURES FOR EVALUATING THE EFFECTIVENESS OF OIL-DISPERSING CHEMICALS

General

This is a simple but a quick method for evaluating the effectiveness of oil dispersing chemicals on a particular sample of oil and is useful in obtaining preliminary data on the performance of the chemical in dispersing the oil. It involves the use of a multiple stirrer, an apparatus employed in water and sewage treatment plants for determining chemical dosage necessary for solids removal. A predetermined amount of chemical is applied on floating oil and then agitated. The effectiveness of the chemical is determined by measuring the amount of oil that is dispersed.

Procedure

- (1) Place a beaker containing 1000 ml water at 25°C (77°F) under the stirrer so that the paddle blade is submerged about $\frac{1}{2}$ -inch below the water surface.
- (2) Pour 10 ml of the oil sample on the surface of water. The oil should be added slowly from a pipette so that it will not spread out rapidly and stick to the beaker walls.

(3) Add 2 ml of the chemical dispersant (undiluted or suitably diluted if viscous) dropwise to cover the entire surface of the oil.

(4) Start the stirrer operating at 100 rpm and continue stirring for 2 minutes.

(5) At the end of 2 minutes, stop the stirrer. Immediately insert a sampling tube ($\frac{1}{4}$ -inch diameter glass tubing connected to a tygon tubing) into the beaker and withdraw a sample of oil-water mixture by siphon from the bottom of the beaker.

(6) Discard the first 50 ml of the mixture and then collect 200 ml in a graduate cylinder.

(7) The amount of the dispersed oil can be measured by the chloroform extraction-spectrophotometric method as described in the S.E.T. Procedure.

Notes on the Procedure

The results obtained from tests with the multiple stirrer will give some preliminary indication of how effectively a particular sample of crude or fuel oil will respond to the treatment with the dispersant. Also, it is possible to determine the approximate dosage required to disperse and emulsify the oil.

Although the method is somewhat crude, it offers

several advantages:

- (a) Preliminary data concerning dosages and relative effectiveness of the dispersant can be obtained very quickly.
- (b) Behaviour of the oil treated with the dispersant can be observed and studied very easily, for example, stability of dispersant-oil emulsions.
- (c) Least effective dispersants can be eliminated at once.
- (d) Comparative tests can be performed simultaneously on several samples of dispersants.
- (e) Tests are very simple and do not require sophisticated equipment or methods.
- (f) Tests can be conducted under controlled conditions, notably temperature and water quality.

These tests also have some limitations and therefore the data obtained must be treated accordingly. Some of these are as follows:

- (a) A good portion of the dispersant may not come into contact with the floating oil during the test but instead, it may dissolve into the water before reacting completely with the oil.
- (b) It is difficult to evaluate the dispersing qualities of some dispersants which performed very well under constant

agitation but quickly reformed into a slick the moment the agitation ceased.

- (c) A large portion of oil will adhere to the test equipment and thus reducing significantly the amount of test sample available for treatment with the dispersant.
- (d) Test samples are likely to be subjected to a much greater amount of agitation than that under actual field applications.

APPENDIX II

TESTS FOR AGENTS FOR SINKING OIL

Screening

Sinking of oil is considered to be unwanted and unwarranted in most situations. Although sinking of oil is commonly done with some steroid treated mineral, it may be done chemically. Therefore, chemical sinking of oil must be monitored in any management program. The following is a preliminary screening method developed by the laboratory of the California Water Quality Control Board to determine the relative amount of oil and dispersant that sinks in seawater after treatment with an OSTA. (Oil spill treating agent)

- (1) Fill an Imhoff cone to the one-litre mark with filtered tap water.
- (2) Pour 25 mls of oil onto the surface of the water. Exact amounts of the oil used may be determined by weighing the bottle before and after application.
- (3) Slowly add 5.0 mls of OSTA in a fine stream contacting as much oil as possible.
- (4) Let stand quiescent for one hour to allow the material to sink.
- (5) Determine the settleable material in ml.

Quantitative

A more sophisticated method has been investigated by the same laboratory. This method will determine only the quantity of oil that sinks. Only those dispersants sinking oil in the screening test need be analyzed by this latter method as described below:

- (1) Add one litre of tap water into a one or two-litre separatory funnel. The temperature must be $20^{\circ} + 0.5^{\circ}$ C.
- (2) Place a small thistle tube into the separatory funnel, so that it extends $\frac{1}{2}$ -inch below the water surface.
- (3) Add 25 mls of the oil to be tested to the surface of the water.
- (4) Add 5.0 mls of the dispersant in a fine stream contacting as much oil as possible.
- (5) Allow to stand quiescent for one hour.
- (6) Move the thistle tube so that it extends half-way into the separatory funnel. Drain the oil and dispersant mixture at the bottom of the funnel into a second one-litre separatory funnel. Wash any oil remaining in the bottom of the initial separatory funnel with 8-10 ml portions of chloroform through the thistle tube into the second separatory funnel.
- (7) Add approximately one litre of deionized water to the

second separatory funnel and shake gently. Drain the chloroform layer through a funnel containing anhydrous sodium sulphate into a 100-ml volumetric flask. Wash the separatory funnel with 3-10 ml portions of chloroform combining the extracts.

(8) Dilute to 100 ml with chloroform. Make any dilutions necessary and determine oil concentrations spectrophotometrically using suitable wavelengths from standard curves of absorbance vs. oil concentrations.

(9) Calculate the percent of oil that sank.

APPENDIX III

MINISTRY OF THE ENVIRONMENT RECOMMENDATIONS

REGARDING BIOASSAY TESTING OF OIL

SPILL TREATING AGENTS

INTRODUCTION

Growing concern relative to the environmental effects of accidental spills of oil has led to increasing interest in the effectiveness and safety of oil spill control agents. Dispersants are likely to play a significant role as one means of dealing with smaller spills of oil for sections of the Great Lakes and interconnecting channels where extensive industrialization and shipping activity prevail.

In this report, the manner in which acute toxicity bioassay can be performed on oil dispersants and combinations of oil plus dispersants is detailed.

In all cases, the Standard Methods for the Examination of Water and Wastewater, 13th edition (1) (See Appendix IIIA) shall be the reference for all quality control measures necessary in running and tabulating an acute toxicity bioassay.

Such essential details as acceptable levels of mortality in fish stocks, acclimation, feeding, weight of fish per unit volume of toxicant and calculation of the tolerance level (T.L.) values are all provided in the above volume.

In the application of the acute toxicity bioassay to oil and oil spill treating agents (O.S.T.A.), certain procedural modifications are necessary. These modifications have been closely aligned and integrated with changes recommended by other workers (2, 3, 4, 5) so that all data will have a common foundation for ease of comparison.

BIOASSAY TEST PREPARATIONS

Standard Reference Toxicant

To ensure consistency in the assays carried out by different workers and to detect any changes in the condition of the test animals which might lead to different results, reference bioassays should be carried out using reagent grade dodecyl sodium sulphate (commonly known as sodium lauryl sulphate) in addition to the usual control tests. A stock solution of dodecyl sodium sulphate is prepared by dissolving 1,000 gms of D.S.S. per 500 mls of standard dilution water. This solution should be prepared

immediately prior to use. When all conditions of the bioassay are met, results of tests made in different areas and by different investigators will be comparable and will serve to indicate the relative toxicity of different dispersant and dispersant-oil mixtures. Results of all bioassays should be expressed as TL-50 values.

Bioassays of each product under study should include tests to determine the median tolerance limit of the dispersant alone and in mixtures with oil in the ratio of 10:1 (oil:dispersant) with each of two crude oils and the fuel oil recommended by the Ministry for use in the bioassays.

Test Organisms

Because of its almost universal distribution in Ontario, the fathead minnow (Pimephales promelas Rafinesque) should be used for fresh water bioassays whenever possible. The rainbow trout (Salmo gairdneri Richardson) may be useful as an optional test species owing to its importance as a prized game fish. For each species under study, specimens should be of the same age and size range and should be obtained from the same source for any test series. Fish heavier than 1.5 grams should not be used because of their

comparatively high oxygen consumption.

Standard Dilution Water

Synthetic fresh water for acclimation, holding and testing may be prepared according to the following formula: Bubble CO₂ through 10 litres of distilled water containing 2.0 gms of CaCO₃ in order to convert it to a clear solution of Ca(HCO₃)₂. Dilute this solution with 20 litres of distilled water and add 30 mls of a mixed salt solution. Any multiple of these volumes and weights may be used to secure desired volumes of the standard water. The mixed salt solution is prepared by dissolving 23.4 gms NaCl, 73.9 gms MgSO₄·7H₂O and 4.4 gms K₂SO₄ in distilled water and making up to one litre. All distilled water should be from a glass still or a tin-lined still to insure the absence of metal ions.

The pH of this water should be in the range of 7.6 to 7.8. Adjustments are made by the addition or removal of CO₂.

Acclimation and Preparation of Test Animals

The test animals should be acclimated for at least 14 days in the standard fresh water. Groups of fish having more than 20 percent mortality in the first 48 hours and more

than five percent mortality thereafter should be discarded.

During acclimation all species are fed a balanced diet. A homogenate of ground clam, 80 percent by weight; dog biscuit or fishmeal, 8 percent, by weight; water, 10 percent by weight and gum arabic crystals, 2 percent by weight, has been found to be satisfactory. This diet - or another formulation selected for use should be frozen immediately after preparation and thawed at room temperature before feeding. Unused, thawed portions must be discarded. These precautions are not necessary if fresh food or fishmeal is used. The fish should be fed twice daily on a demand basis but must not be fed for 48 hours prior to or during bioassay tests. Only those organisms which feed actively and appear to be healthy should be used in the bioassays and any individual injured or dropped while handling must be discarded.

The minnows should be acclimated to a water temperature of $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ($20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for rainbow trout) and a pH of 7.5 and 7.8.

Oxygen levels should be kept above 4 ppm, preferably between 5 and 6 ppm during the acclimation period. All

tests in a series should be run with the same batch of oil and with organisms from the same group. Specimens should be of uniform size and age and of the same species.

BIOASSAY PROCEDURE

Each test should consist of five replications of each concentration of the material under study. Concentrations of the materials are to be at quarter points in a logarithmic series; for example, concentrations might be 10 ppm, 18 ppm, 32 ppm, 56 ppm, 100 ppm. In addition to these five concentrations, five control jars and a standard reference test series at five different concentrations should be set up. This makes 35 jars in all.

The test should be carried out using 4-litre (approximately 1 gallon) glass jars measuring approximately 22.5 cm high, 15 cm in diameter and 11 cm in diameter at the mouth. The jars should have screw top lids and be lined with a non-toxic plastic film (e.g., polyethylene bags). In carrying out the test, two litres of the synthetic dilution water aerated to saturation should be added to each of the jars. For ease and accuracy in adding the two litres, an automatic dispensing pipette of two litres capacity is

recommended.

The test jars containing two litres of the standard water are placed on a reciprocal shaker. The desired amount of petroleum product under test may be added directly to each test jar with a calibrated syringe. It is important that all tests in a bioassay series should be run with oil from the same container. Dispersant standard water stock or the undiluted dispersant is then added with a pipette or by means of a calibrated syringe. The test jars should be tightly capped and shaken for five minutes at approximately 315-333 three-fourths inch strokes per minute on the reciprocal shaker.

The shaker platform should be adapted to firmly hold six bioassay jars. At the completion of the shaking, the jars are removed from the shaker and placed in a constant temperature water bath or room, after which the lids are removed and two fish added.

Mixtures of oil and the chemical or dispersant under consideration are tested in the ratio of 10 parts oil by volume to 1 part dispersant by volume. Note that the results are expressed in millilitres total product per litre

of medium and that a 96-hour TL50 of 0.506 mls total product consists of approximately 10 percent dispersant (0.046 mls/l) and approximately 90 percent oil (0.460 mls/l).

Since oils and dispersants contain volatile materials, many of which are toxic, and since the toxicity of some of the water soluble fractions of oil and its degradation products are modified by oxidation, special care must be taken in the oxygenation of test solutions. Gentle aeration to maintain D.O. and to insure uniformity should be provided. The dissolved oxygen content of the solutions should not fall below 4 ppm for fathead minnows or 6 ppm for rainbow trout during the bioassay. In order to limit the removal of volatiles, aeration should be at the rate of 100 ± 15 bubbles per minute supplied from a 1 ml serological pipette having an inside diameter of 1 mm. At this rate, and with the proper weight of fish, the D.O. concentration in the test jars can be properly maintained over a 96-hour period. Dissolved oxygen measurements must be taken at least daily.

Standard Reference Test Oils

Oil used in bioassays and other test procedures

should be as it comes from the well or as it is produced and shipped in Ontario. Small portions used in bioassays should be transported and stored in sealed containers to prevent the loss of volatiles and other changes. For ease in handling and use, it is recommended that 100-ml glass containers be used and minimum of 40 containers of each grade or kind of oil under test be available.

Gastight calibrated syringes of 0.001, 0.01, 0.1, 1.5, 10, 30, and 60 ml capacity should be used to add the calculated amounts of the test materials to each test jar. These should have teflon tipped plungers. Upon completion of the dosing, unused oil in each open container should be disposed of to prevent its use at a later date when certain volatile materials may have been lost. Dispersants used for bioassays should be kept sealed when in storage. Oil dispersant ratios employed in the tests should be in accordance with manufacturer's recommendations. Generally, the ratio is one to ten by volume.

Test Oils

The fuel and crude oils used for tests shall conform to the following specifications:

- (1) No. 6 fuel oil per ASTM Specification D-396-67.
- (2) Alberta crude oil having the general characteristics typical of the Leduc field crude oil as shipped into Ontario via Interprovincial Pipeline Co. Ltd.
- (3) Alberta crude oil having the general characteristics typical of the Lloydminster field crude oil as shipped into Ontario via Interprovincial Pipeline Co. Ltd.

DEGRADABILITY PROCEDURE

In order to assess the degradation of O.S.T.A., acute toxicity bioassays are carried out on aged samples. Having determined the 96-hour TL_m of fathead minnows or other test organism to the O.S.T.A., a solution or dispersion of the O.S.T.A. equivalent to ten times the 96-hour TL_m concentration is made up in standard fresh water. This solution is aerated at the rate of 100 ± 15 bubbles per minute supplied from a three millimetre inside diameter glass tube at a temperature of 15°C and for a period of twenty days.

At the end of the sixteenth day of aeration, test organisms are introduced into the solution and the acute toxicity of the solution to the organisms is monitored for a further 96-hour period.

REFERENCES

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- (4) United States Dept. of the Interior - F.W.P.C.A. Interim Toxicity Procedures for Oil Spill Control Chemicals, 1968.
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APPENDIX IIIA

BIOASSAY PROCEDURE APHA STANDARD METHOD

INTRODUCTION

The bioassay procedures described here are intended for use in industrial and other laboratories in evaluating the toxicity of wastes and other water pollutants to fish. These practical tests can be used to determine whether or not a waste or waste component is markedly toxic and, if toxic, the degree of toxicity. They will serve also as the basis for judging whether or not a waste can be discharged at a given rate without causing direct injury to fish and other organisms in the receiving water.

The value of such biologic tests of waste quality, in connection with the control of waste disposal and treatment, is being increasingly realized. Chemical examination of complex industrial wastes alone usually does not yield sufficient information. Many of their various toxic components cannot be readily detected, separated, and measured by chemical means. Moreover, the degree of toxicity of each of these numerous substances and mixtures of chemicals is not known. The toxicity of wastes can be greatly influenced by interactions between their individual components and the dissolved minerals present in widely varying amounts in

receiving waters. Different kinds of fish are not equally susceptible to toxic substances, and much of the pertinent published information is based on experiments with hardy species only. Therefore, the toxicity of an industrial waste to local fish in their natural medium must be detected and evaluated directly through biologic tests under appropriate experimental conditions.

Death due to deficiency of dissolved oxygen (DO) in polluted water should be distinguished from death due to toxicity. To detect and evaluate the direct lethality of the wastes only, adequate DO must be maintained during toxicity tests.

Reasonable uniformity of experimental procedure and of the manner of presentation of results is essential; widespread adoption of uniform methods will promote the accumulation of comparable data. However, rigid standardization of experimental material and conditions is not desirable, for it would tend to defeat the purpose of practical tests. Strict comparability of test results could be achieved only by sacrificing much of their relevance and applicability to practical problems in specific localities. Experimental water (water used as a diluent) and test animals best suited

to the purpose of each bioassay should be selected. With respect to other features, the tests can be more or less uniform. It is believed that the standardization of experimental conditions and procedures described here will insure adequate uniformity, reproducibility, and general usefulness of the bioassay results, without interfering unduly with the adaptability of the tests to local circumstances and problems.

Selection of method: The basic Routine Bioassay Method constitutes the simplest procedure. It is widely applicable, being suitable for the detection and evaluation of acute toxicity which is not associated with excessive oxygen demand and is due to substances that are relatively stable and are not extremely volatile. The Routine Bioassay Method is designed so that surface absorption of oxygen from the atmosphere plus some oxygen from the diluent generally provides an adequate amount of DO for the fish during the test period. Many industrial effluents and some chemicals have a high chemical or biochemical oxygen demand, which may cause oxygen depletion in test solutions. Although it is usually necessary to use artificial supplies of air or oxygen, DO must nevertheless be maintained at levels adequate for the

test fish.

Uncontrolled aeration with compressed air has been generally unsatisfactory in bioassays of industrial effluents. Small additions of air will not ordinarily maintain the necessary quantities of DO, whereas vigorous aeration may drive off volatile toxicants and may greatly speed up biologic oxidation.

Three methods of maintaining adequate DO are described under Modifications of Routine Bioassay Method: (1) controlled artificial oxygenation of test solutions; (2) initial oxygenation of the diluent; and (3) renewal of test solutions.

Renewal of test solutions should also be employed whenever there is evidence or expectation of a rapid change of toxicity of test media. Such a change usually can be detected by testing solutions or waste dilutions which have stood in the test vessels for about 2 days and in which test animals exposed to the fresh media have died during this period. Serious volatility, instability, or detoxification of important constituents is indicated when the recorded average survival time of the test animals in a fresh medium is much less than the survival time in the corresponding

older, used medium, if adequate DO is present throughout both tests.

Another modification of the routine bioassay method that may be useful in some instances is prolonging the test period beyond 96 hr. This is sometimes done when the material tested is of a low order of toxicity or when 24-, 48-, and 96-hr results indicate a major increase in mortality with time of exposure.

When the experimental data are reported, any deviation from the basic routine procedure should be described in order to facilitate interpretation of test results and their significance.

ROUTINE BIOASSAY METHOD

1. General Discussion

1.1. Principle: The prescribed measure of acute toxicity is the median tolerance limit (TL_m) or the concentration of the tested material in a suitable diluent (experimental water) at which just 50 percent of the test animals are able to survive for a specified period of exposure. This exposure period may be 24, 48, or 96 hr. At least the 24- and 48-hr TL_m values should be determined and recorded whenever the toxicity is sufficiently pronounced to permit their determina-

tion.

Concentrations of dilutions of liquid industrial wastes (aqueous solutions, suspensions, and emulsions of complex or unknown composition) are expressed as percent by volume. For example, a 10 percent dilution, or a TL_m of 10 percent, equals 1 part of wastewater in 9 parts of diluent water. Concentrations of solutions of nonaqueous wastes and of individual chemicals (solids, liquids, or gases) are expressed in terms of milligrams per litre, or parts per million by weight of the substance added to the experimental water.

To evaluate the toxicity, different concentrations are tested so that the concentration lethal to 50 percent of the test animals within each prescribed test period can be found or estimated by interpolation. DO concentrations well above the minimum tolerable level are maintained throughout the course of each test, but unduly rapid reaeration of the test medium, which can result in speedy removal of important volatile constituents, is avoided.

1.2. Interference: Extreme volatility, instability, and rapid detoxification of waste components and chemicals tested, and also excessive oxygen demand, can render the

routine bioassay method inapplicable, or can cause serious bias of the results. (See "Selection of Method" above and Sec. 9.2 below.)

1.3. Sampling and storage: Samples of industrial or other effluents which are not constant in their composition should be collected at different times and should not be unnecessarily combined to make composite samples, because knowledge of the maximal toxicity of a variable effluent often is required in connection with the control of waste disposal in flowing waters, rather than knowledge of the average toxicity.

Samples of industrial or other effluents should be stored in completely filled, stoppered bottles at a fairly uniform temperature not greatly exceeding the intial temperature. If the waste may contain organic matter subject to bacterial decomposition, the samples should be refrigerated (without freezing) and held at a temperature between 0° and 4°C. Duration of storage prior to testing should be kept to a minimum.

2. Apparatus

2.1. Test containers: The size and shape of the vessels or aquaria, hereafter referred to as test containers, in which fish are exposed to the test media, have not been

standardized. The size depends on the required volume of test medium, which in turn depends on the number and size of fish used in each test (Section 5.6.). However, the depth of the vessels must be more than 6 in. (Section 5.2.). Test containers should be of glass and should be chemically clean. For tests with fish of ordinary size, 2 to 3 in. in length, the containers may be wide-mouth glass bottles or cylindrical glass jars 10 to 12 in. in diameter, 12 in. or more high, with a capacity of about 4.5 to 5 gal (17 to 19 litres). Smaller or larger glass jars, or rectangular aquarium tanks, may be more suitable for tests with smaller or larger fish.

Six to twelve test containers often are needed for performing a bioassay most efficiently.

2.2. Testing laboratory: An ordinary heated or air-conditioned laboratory room with thermostatic controls suitable for maintaining prescribed test temperatures (Section 5.1.) will generally suffice for the conduct of bioassays with warm-water fish. A specially insulated constant-temperature room or a large water bath equipped for precise temperature control and good circulation of air or water is usually preferable, and such a facility may be necessary for conducting bioassays with cold-water fishes.

2.3. Acclimatizing tank: The aquarium in which the test animals are acclimatized and prepared for use in toxicity tests (Section 3.6.) usually is a rectangular tank with glass sides and a capacity of 15 to 50 gal, its size depending on the size and number of fish to be used. Because the water in the acclimatizing tank must approximate the test temperature, this tank must be placed in a room with suitable, constant temperature, or it must be equipped with thermostatic devices. Thermostatic equipment must be selected with care to avoid contact of the water with harmful metals, such as copper and copper alloys, zinc, and cadmium. Facilities must be provided also for adequate aeration with compressed or pumped air released near the bottom of the tank from several air diffusion stones or dispersers.

3. Selection and Preparation of Test Animals

3.1. Kinds of fish to be used: The test fish should be a species adaptable to the laboratory conditions of temperature, feeding, and handling. Availability of an adequate supply of healthy fish of desirable uniform size may be the major factor governing selection of the species to be used. Information on species which have been used successfully is given in the literature.^{1, 9, 12}

If available, fish species which are deemed important locally should be given preference. If circumstances necessitate the use of some other kind, such as one of the common aquarium fishes (guppies, goldfish, etc.), for routine bioassays, or if one of these aquarium fishes is chosen in order to insure uniformity of test animals at all times with respect to age, size, nutritional and temperature history, etc., comparative tests should be performed, using appropriate toxicants, to relate the sensitivity of the selected species to those of locally important game and food fishes.

Although any fresh-water or marine fish species that suits the purpose of the investigation may be used, species belonging to any of the following widely distributed and important fresh-water families are particularly recommended, and one or more of these should be selected unless there is good reason for making a different choice:

Centrarchidae (sunfishes, basses, crappies)

Salmonidae (trouts, charrs, salmons)

Cyprinidae (true minnows)

Catostomidae (suckers)

For tests relating to estuarine pollution problems and some others, some species belonging to the families Gas-

terosteidae (sticklebacks) and Cyprinodontidae (killifishes, top minnows) can be outstandingly suitable test animals, because of their tolerance of wide variations of water salinity, their abundance in many coastal waters, their small size, etc. However, species of the family Cyprinodontidae may be much more resistant to many toxicants than most of the other recommended forms. Marine species of the genus *Fundulus* have been widely used as experimental material.

3.2. Identification: Fish used for each individual toxicity evaluation must all be of the same species. They should be identified at least as to genus, and preferably as to species. The correct scientific name should be stated when the test results are reported.

3.3. Source: Test fish may be obtained from any single, common source (hatchery, lake, stream). They should all be collected and brought to the laboratory for acclimatization at about the same time.

3.4. Size: The largest fish in an individual bioassay should be not more than 1.5 times the length of the smallest specimen used. Small specimens, averaging less than 3 in. in length, generally are the most convenient and desirable test animals.

3.5. Stocks: Stocks of test fish may be kept initially in any suitable enclosures or containers (small ponds, live-boxes, screened pens, concrete or wooden tanks, or glass aquaria) and in any water of suitable quality and temperature in which they remain in good condition until needed. Care and feeding of stock animals are discussed by Doudoroff and colleagues.⁵

3.6. Acclimatization and feeding: The test animals should be acclimatized for at least 1 week (preferably 10 days or longer) to laboratory conditions similar to those under which the tests are to be performed, with regard especially to the temperature and chemical properties (or source) of the water.

The fish should be fed at fairly regular intervals, at least three times a week and preferably daily, during the acclimatization period, but should not be fed for a period of about 2 days before they are used in a test.

3.7. Fitness: The incidence of specimens dying or becoming seriously diseased in the acclimatizing aquarium during a period of 4 days immediately preceding a test must be less than 10 percent. Otherwise, the test animal lot should be deemed unfit for use until the incidence of disease

and the mortality rate decline sufficiently. Test specimens must show no symptoms of disease or abnormalities of appearance or behavior at the time of their transfer to test containers.

4. Section and Preparation of Experimental Water (Diluent)

4.1. Usual source of experimental water: The experimental water to be used as a diluent and acclimatizing medium should be obtained from the body of water which receives the waste under investigation. When the toxicity of a waste alone is to be determined, the water should be obtained at a point where there is no pollution or contamination with waste from any source.

When a stream water receiving the waste to be tested is subject to previous contamination with other wastes, the toxicity of the test waste in conjunction with previous contaminants must be considered in judging safe rates of discharge. For evaluation of this resulting toxicity, it is necessary to use experimental water obtained immediately upstream from the point of discharge of the waste to be tested, but outside the zone of its influence. Such an evaluation is possible only when the test animals can be held successfully in the diluent water.

4.2. Substituted water: If uncontaminated experimental water cannot be supplied from the body of water under consideration, water of similar quality, with respect to its dissolved-mineral content, should be obtained from another source, or else prepared by adding appropriate chemicals to a natural water of suitable quality, which may first be diluted, if necessary, with distilled or demineralized water of assured purity. As a general rule, the calcium, magnesium, sulphate, and dissolved-solids content of this substituted water should not differ by more than 25 percent from that of the water receiving the waste tested. It is advisable to adjust the pH, alkalinity, and hardness of the substituted water to match those of the receiving water as closely as practicable. This is especially necessary when effluents are known to contain metal salts, cyanide complexes, ammonium compounds, or other chemicals the toxicity of which is known to be greatly affected by changes in these characteristics. When wide variations occur in quality characteristics of receiving waters, it is desirable to determine the toxicity of the waste material at the upper and lower limits of the range.

4.3. Modification of water for use as diluent: When

uncontaminated water is used as a diluent it should be adjusted to the test temperature and well aerated with dispersed compressed air. Excessive amounts of noncolloidal suspended matter should be removed by settling or filtration.

When a contaminated water is employed as a diluent, delay of its use, unnecessary aeration, and other treatment which can result in serious alteration or removal of the pollutants present, should be avoided.

5. Other Prescribed Experimental Conditions

5.1. Test temperatures: The tests should be performed at a uniform temperature between 20° and 28°C when so-called warm-water fish (e.g., Centrarchidae and most Cyprinidae) are used as test animals, or between 12° and 18°C when cold-water fish (e.g., Salmonidae) are used.

It is desirable to hold the temperature as closely as possible to the selected and reported value. For reliable results the overall range should not exceed 4°C.

5.2. Depth of liquid in test containers: The average depth of the liquid should be uniform in all parallel tests and should never be less than 6 in. The purpose of the latter restriction is to limit the rate of escape of any gaseous or other volatile components, which varies with the ratio of the

exposed surface area to the volume of the liquid.

5.3. Dissolved oxygen content of liquids tested: The DO content of test solutions, or dilutions, should not fall below 4 mg/l when warm-water fish are used as test animals, or 5 mg/l when cold-water fish are used.

5.4. Aeration and oxygenation of liquids tested: Re-aeration of the liquid in a test container occurs at the still surface exposed to the atmosphere. When the rate of absorption of oxygen from the air is not great enough to maintain the prescribed DO concentration, other methods for regulation or maintenance of the oxygen content must be employed (see Modifications of Routine Bioassay Method). Unregulated artificial aeration of waste samples, and dilutions, by dispersed compressed air or other means, is not permissible unless no gaseous or volatile components, which may be driven rapidly out of solution, are present. Thorough aeration is an approved treatment for some wastes before discharge, but the toxicity of a waste or waste dilution which has been aerated is often less than that of an unaerated sample.

5.5. Number of test animals: At least ten animals should be used for testing each experimental concentration of

the substance or waste under investigation, if final conclusions are to be based on the outcome of the test. These animals may be divided equally among two or more test containers with solutions, or waste dilutions, of the same concentration. Indeed, duplication of each test is desirable. Fewer than ten animals may be used in preliminary tests (Section 6.1a.), to determine the range of concentrations which should be tested with the larger number of fish in the routine test procedure (Section 6.1b.).

5.6. Total animal weight and liquid volume: The weight of all fish in a test container should not exceed 2 g per litre of liquid medium. Preferably, it should be about 1 g, or less, per litre of liquid, especially when the average weight of the fish is not much more than 2 g each. Sometimes the weight of fish which can be held successfully in a given volume of liquid may be further restricted by the available oxygen supply and related requirements.

6. Procedure

6.1. Procedural elements: The concentration of a test waste or other substance fatal to 50 percent of the test animals in a specified exposure period, under the prescribed experimental conditions, sometimes can be found directly by

experiment, but such direct determination of the TL_m is not often practicable or advantageous. It is sufficient to record the percentages of test animals surviving at concentrations somewhat higher and lower than the TL_m , so that it can be estimated by interpolation.

a. Exploratory tests: When testing effluents of completely unknown toxicity, much time and effort may be saved by conducting small-scale exploratory bioassays to determine the approximate range of concentration of the waste which should be covered in the full-scale tests. In these preliminary tests, solutions are prepared over a wide range of concentrations; for example, 100, 10, 1, and 0.1, or 100, 32, 10, and 3.2 percent of the effluent by volume.

An industrial effluent or a saturated solution of a chemical of uncertain toxicity can be tested without any dilution only if it has an adequate DO content. If found to be deficient in DO, it must be diluted with well-aerated experimental water until the required persistent DO concentration (Section 5.3.) is attained, before introduction of the test animals. If at least half of the test animals die during the test while the DO concentration remains adequate, and all or nearly all controls (Section 6.3.) survive, measur-

able acute toxicity of the pollutant is indicated. If no fish are killed in 24 hr, the test should be continued for 48 or 96 hr. If toxicity cannot be demonstrated and evaluated by the routine method, a modification may have to be tried before concluding that no acute toxicity can be detected and measured.

In the exploratory tests two or more fish are placed in an appropriate volume of each of the test solutions. For two average-size test fish (2-3 in. long) 2 litres of test solution in 1-gal wide-mouth glass bottles are usually adequate. The duration of the exploratory tests depends on the results obtained. Usually observations overnight or for 24 hr will indicate the percentages between which the concentrations for the full-scale tests should be selected. When the test materials have low toxicity or act slowly, or when the waste must be diluted to insure adequate oxygen, it may be necessary to continue the test for 48 or 96 hr to demonstrate toxicity and to determine the concentration range to be tested. Usually the test range to be used in the full-scale routine tests falls between the highest concentration at which all fish survive for 24 hr and the lowest concentration at which all or most fish die in the same period.

Exploratory tests can also indicate whether excessive oxygen depletion or loss of toxicity in test solutions is such that modifications of the routine bioassay method are necessary.

b. Full-scale tests for toxicity evaluation: When the range in concentration or test range to be covered by the full-scale tests has been determined by exploratory bioassays, the toxicity of the pollutant is measured by testing several concentrations within the limits of this range. A series of at least 4-6 such concentrations should be tested in order to make possible a sufficiently precise estimation of the TL_m .

6.2. Choice of concentrations to be tested: Although TL_m may be determined by using any appropriate concentrations of the substance or waste to be assayed, it has been found best and most convenient to use the logarithmic series of concentration values given in Table 1. These values can represent concentrations expressed as percent by volume, as milligrams per litre, or as parts per million by weight. They may be multiplied or divided, as necessary, by any power of 10. For example, the two values in the first column may be 10.0 and 1.0 as shown, or they may be 100 and 10, or 1.0

and 0.1, with the values in the other columns changed accordingly. The series of values, 10.0, 5.6, 3.2, 1.8, and 1.0 percent, are evenly spaced when plotted on a logarithmic scale. Generally five such points are adequate for obtaining the desired information on the variable effluents from most industries. A higher degree of precision can often be obtained by using the intermediate concentrations listed in Col. 4; namely, 7.5, 4.2, 2.4, and 1.35 percent. The concentrations in Col. 5 are used only in very unusual circumstances.

Some investigators prefer other, similar series of concentrations, such as that presented in Table 2. The values shown in Col. 1 usually are used first. If additional tests are deemed advisable for reducing the intervals between the test concentrations, the appropriate values from Col. 2 may be used. The reason for selection of these concentration values will be apparent when their logarithms, given in the right-hand column, are considered. Other series of test concentrations can be equally satisfactory, but a logarithmic series is always advantageous and should be approximated. A more detailed description of the use of logarithmic series of concentrations is given by Doudoroff, et al.⁵

TABLE 1

GUIDE TO SELECTION OF EXPERIMENTAL CONCENTRATIONS, BASED ON
PROGRESSIVE BISECTION OF INTERVALS ON LOGARITHMIC SCALE

Col. 1	Col. 2	Col. 3	Col. 4	Col. 5
10.0				8.7
			7.5	6.5
		5.6		4.9
			4.2	3.7
	3.2			2.8
			2.4	2.1
		1.8		1.55
			1.35	1.15
1.0				

TABLE 2

**GUIDE TO SELECTION OF EXPERIMENTAL CONCENTRATIONS,
BASED ON DECILOG INTERVALS**

Concentrations		Log of Concentration
Col. 1	Col. 2	
10.0		1.00
	7.94 (or 7.9)	0.90
6.31 (or 6.3)		0.80
	5.01 (or 5.0)	0.70
3.98 (or 4.0)		0.60
	3.16 (or 3.15)	0.50
2.51 (or 2.5)		0.40
	1.99 (or 2.0)	0.30
1.58 (or 1.6)		0.20
	1.26 (or 1.25)	0.10
1.00		0.00

The magnitude of suitable intervals between the test concentrations used for establishing a TL_m by interpolation cannot be definitely prescribed. It depends on the required degree of precision (which varies with the planned use of the test results) and on the nature of the experimental data. When two test concentrations, one above and one below the TL_m , have proved unquestionably lethal to some (about 20 percent or more) but not to all of the test animals, determination of survival percentages at intermediate concentrations generally is not essential. Otherwise, the estimate of TL_m can often be markedly improved by reducing the intervals between the test concentrations. However, the testing of more concentrations may not be justifiable when the increased reliability of the estimate thus achieved can have no practical import. The intervals between the concentrations included in the first column of Table 2, and those included in the first three columns of Table 1, are believed to be sufficiently small for most industrial effluents. Narrower and wider intervals are recommended only when they are clearly advantageous and adequate.

In preparing test solutions of highly toxic compounds, it may not be feasible to add measured quantities

TABLE 3

DILUTIONS FOR VARIOUS TEST SOLUTION CONCENTRATIONS

Test Solution Concentration Desired		Strength of Stock Solution--g/l				
percent	ppm or mg/l	ppb or ug/l	100	10	1	0.1
		Volume (ml) to Be Diluted to 1 Litre				
1.0	10,000		100			
0.56	5,600		56			
0.32	3,200		32			
0.18	1,800		18			
0.10	1,000		10	100		
0.056	560		5.6	56		
0.032	320		3.2	32		
0.018	180		1.8	18		
0.010	100		1.0	10	100	
0.0056	56		5.6	56		
0.0032	32		3.2	32		
0.0018	18		1.8	18		
0.0010	10		1.0	10	100	
0.00056	5.6		5.6	56		
0.00032	3.2		3.2	32		
0.00018	1.8		1.8	18		
0.00010	1.0	1,000		1.0	10	100
0.000056	0.56	560		5.6	56	
0.000032	0.32	320		3.2	32	
0.000018	0.18	180		1.8	18	
0.000010	0.10	100		1.0	10	
0.0000056	0.056	56			5.6	
0.0000032	0.032	32			3.2	
0.0000018	0.018	18			1.8	
0.0000010	0.010	10			1.0	

directly to the test aquaria. Table 3 is useful in determining suitable quantities of dilutions to be added.

6.3. Control tests: With each test of any concentration of the substance assayed, or with each series of tests of different concentrations tested simultaneously, a concurrent control test must be performed in exactly the same manner as the other tests, using the experimental water (diluent) alone as the medium in which the fish (controls) are held. The DO content of the water in the control test containers is subject to the restrictions applicable to other tests. There should be no more than 10 percent mortality among the controls during the course of any test, and at least 90 percent must remain in apparently good health. Otherwise, the test results cannot be deemed reliable and every simultaneous test should be repeated.

6.4. Preparation of test dilutions of wastes: All dilutions required for a single toxicity bioassay should be prepared with the same sample of waste, portions of which may be stored until needed in completely filled, tightly stoppered bottles at a temperature of 0° to 4°C (without freezing). Duration of storage should be held to a minimum and should be reported, along with the source and nature of

the sample. Any undissolved material present in a waste sample should be uniformly dispersed by agitation before a measured portion of the sample is withdrawn for addition to a measured quantity of water in a test container. The waste then should be mixed thoroughly with the diluent by gentle stirring to insure good dispersion and solution of any undissolved soluble matter. Unnecessary exposure of the waste samples and dilutions to the atmosphere, through violent agitation or otherwise, should be carefully avoided.

6.5. Transfer of test animals: Test animals should be transferred from the acclimatizing aquarium to the test containers as soon as possible but at most within 30 min after preparation of the experimental solutions or waste dilutions. This time interval should be uniform and should be recorded. Test animals should be transferred from one container to another only with small-mesh dip nets of soft material or with wet hands, and should not be allowed to rest on any dry surface or be held out of water longer than necessary. Any specimen accidentally dropped or otherwise mishandled during transfer should be rejected and not used for test purposes until its health and freedom from injury have been established. All the test animals should be selected and graded in advance

according to size to avoid unnecessary handling just before the test.

6.6. Duration of tests: Duration of all tests should be at least 48 hr, and preferably 96 hr. In the event that more than half of the test animals survive for 48 hr at the highest concentration that can be tested properly, the test must be continued for 96 hr. The substance or waste under investigation may be reported as having no acute toxicity only when it has been shown that most of the test animals survive for 96 hr at this maximal test concentration. If toxicity cannot be demonstrated or evaluated by the routine procedure, modifications should be employed before concluding that no significant acute toxicity can be detected and measured.

6.7. Observations during tests: The number of fish which have died in each test container should be observed and recorded exactly 24 and 48 hr after their introduction, and also after 96 hr if the tests are continued beyond the minimum (48-hr) period. The number of fish which are alive but show pronounced symptoms of intoxication and distress, such as loss of equilibrium and other markedly abnormal behavior, also should be noted and recorded. Close observations

of the reactions of the fish during the first 4 to 8 hr may give an indication of the nature of the toxicant and serve as a guide for further tests. Test animals should be deemed "dead" at the time of observation only if respiratory and other movements, either spontaneous or in response to mild mechanical stimulation (prodding the animal or pressing its tail with a glass rod), cannot be readily detected during an observation period of about 5 min. Dead fish should be removed as soon as observed.

Minimum required quantities (samples) of the liquids tested should be removed from the test containers as often as necessary for determination of DO or other instructive chemical tests. To maintain the liquid surface at a uniform level, such samples may be replaced with equal volumes of similar solutions or dilutions, prepared at the same time as those tested and stored in separate containers.

6.8. Feeding of test animals during tests: The fish should not be fed during tests of limited duration (96 hr or less). Feeding tends to increase the rate of respiratory metabolism and increases excretory or other waste products which may influence the toxicity of test solutions.

7. Physical and Chemical Determinations

Determinations that should be made are temperature, DO, and pH. Others, such as alkalinity, acidity, and hardness, may be useful, depending on the nature of the effluent.

Determination of DO is necessary to detect any fish mortality caused by oxygen depletion and to assure control of oxygen during the bioassays. High or low pH values which may cause fish mortality can be readily determined. Changes in pH and other water quality characteristics may have a definite bearing on the toxicity of many substances. Chemical tests may also give indications of the nature of the major toxicants and may help greatly in the application of bioassay results.

Physical and chemical determinations are normally made on test solutions before adding fish and after fish mortality or at the termination of the bioassay. Some determinations, especially for DO, may be needed more frequently. Samples may be siphoned directly from the test aquaria into small DO bottles and other containers.

8. Calculation (Estimation of TL_m)

A TL_m reported may be a concentration at which 50 percent survival actually was observed in a test (if higher

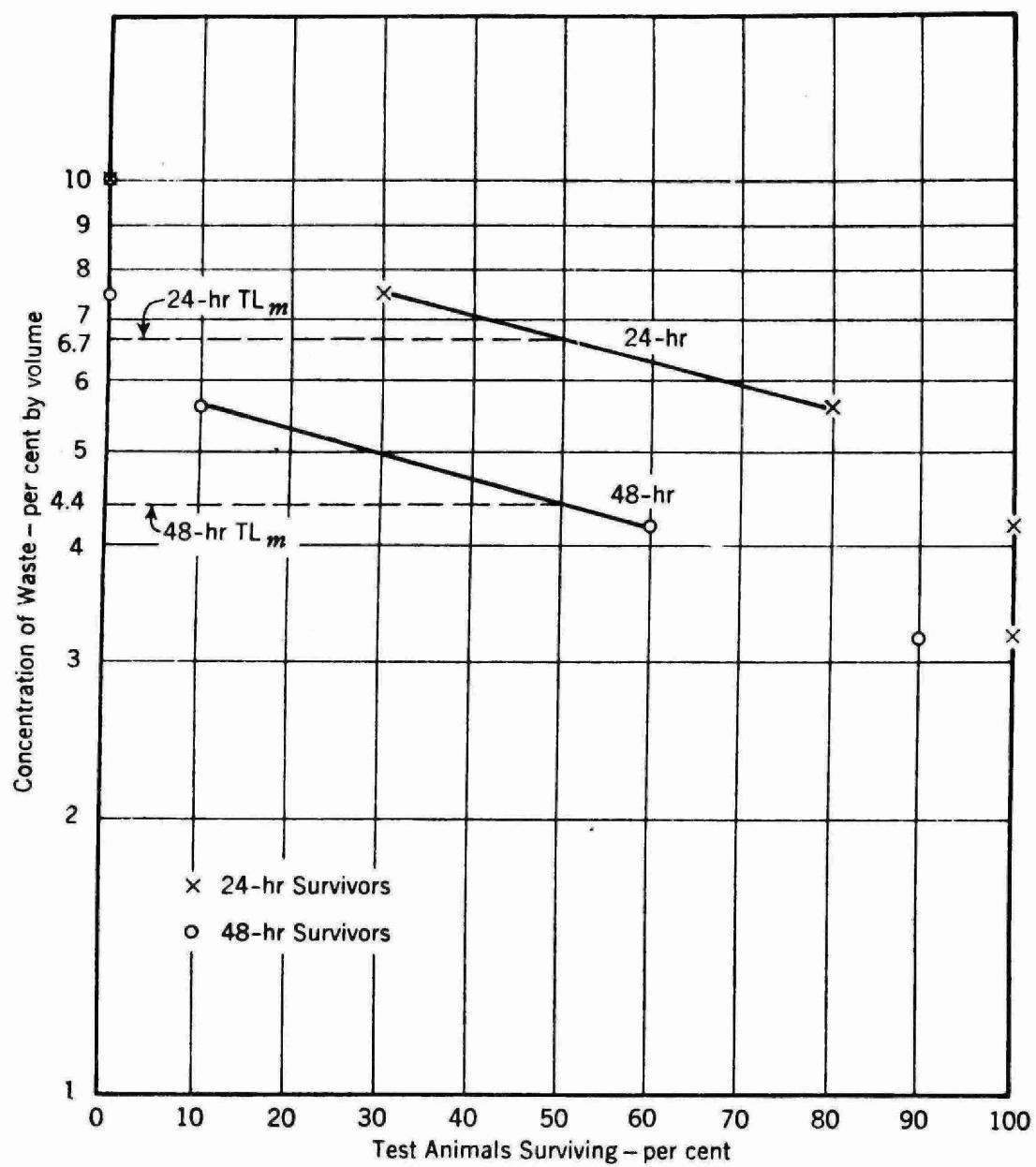
and lower percentages have been recorded for the next lower and higher test concentrations, respectively); or it may be a value obtained by interpolation, based on observed percentages of test animals surviving at concentrations lethal to more than half and to less than half of the test subjects.

The derivation of the median value by interpolation involves merely plotting the experimental data on semi-logarithmic coordinate paper, with test concentrations laid off on the logarithmic scale and survival percentages on the arithmetic scale. Then a straight line is drawn between the two points representing survival percentages at two successive concentrations of the test series which were lethal to more than half and to less than half of the test animals. From the point at which this line intersects the 50 percent survival line a perpendicular drawn to the concentration ordinate indicates the TL_m concentration. This method is referred to as straight-line graphical interpolation.

Figure 1 illustrates the estimation of TL_m values by the straight-line graphical interpolation method. Hypothetical experimental data are presented in the accompanying table, which shows the numbers of animals used for testing, different dilutions of a liquid industrial waste or effluent,

ROUTINE BIOASSAY METHOD

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Experimental Data (Hypothetical) Plotted Above

Concentration of Waste % by. vol.	No. of Test Animals	No. of Test Animals Surviving	
		After 24 Hr	After 48 Hr
10.0	10	0	0
7.5	10	3	0
5.6	10	8	1
4.2	10	10	6
3.2	10	10	9

Fig. 1 Estimation of median tolerance limits by straight-line graphical interpolation

and the numbers surviving after exposure periods of 24 hr and 48 hr. These experimental results are plotted in the figure. The 24-hr and 48-hr median tolerance limits are shown to be about 6.7 and 4.4 percent waste, respectively.

If the difference, or interval, between the two important test concentrations is not much more than 25 percent of the higher value, a rough estimate of the TL_m sufficient for most practical purposes can be obtained even when the observed survival percentages at these concentrations are 0 and 100 percent. At least one higher and one lower concentration always should be included in the series tested, in order to obtain confirmatory data.

Other widely accepted and often more satisfactory procedures include graphical methods which involve fitting a smooth, sigmoid curve to data plotted in the manner described above, or fitting a straight line to data plotted on logarithmic probability paper,⁴ as well as the more refined methods of probits, logits, or angles.⁷

Sometimes the observed survival percentages change little or erratically with progressively increasing concentration of the test substance, even rather large changes of concentration having but little effect on its lethality, for

one reason or another. Under these circumstances it may be necessary to resort to one of the above methods of TL_m estimation rather than the straight-line graphical interpolation method in order to make full use of the available experimental data to achieve satisfactory precision.

9. Precision and Accuracy

9.1. Precision: The precision of toxicity bioassay results obtained by the routine method cannot be explicitly stated. It depends on the degree of uniformity of the test animals with respect to their resistance to the particular toxicant assayed, and on the number of animals used in each test. It is influenced also by the number of concentrations within the test range which are bioassayed and, to some extent, by the method of interpolation (estimation of TL_m) employed. None of these factors has been rigidly standardized, inasmuch as the required precision varies widely with the purpose of the bioassay or the planned use of the test results.

The normal biologic variation of individuals of a species limits the precision of any bioassay method. All individuals do not react identically to a toxicant, and minor differences in their metabolism may, in replicate tests,

cause slight changes in test solution characteristics, such as pH, carbon dioxide, and DO. A precision within 10 percent is the greatest that can be expected under carefully standardized conditions when using ten uniform fish in solutions of a toxicant whose action is not greatly affected by changes in characteristics of test solutions. The precision with toxicants (e.g., metal salts) whose action is greatly affected by changes in test solution characteristics is much less. The use of additional replicate tests and a larger number of test fish may increase the precision.

9.2. Accuracy and interpretation of bioassay results: It should be understood that a routine toxicity bioassay yields only a measure of the toxicity of a substance to a particular, selected group of fish of a certain kind, size, age, and physiologic condition, at a certain time or season, and when the substance is added to water with specific characteristics. The TL_m determined may or may not closely approximate the true median tolerance limit for an entire population of fish under average conditions to be found in their natural habitat. The test animals used in a bioassay usually are not truly representative of an entire population with respect to size, age, environmental history, and

physiologic condition; the TL_m obtained consequently differs, more or less, from the median tolerance limit for the population. However, such "inaccuracy" of a bioassay is not usually to be deplored. In most instances, it is indeed desirable to determine a TL_m for sensitive fish under the most adverse conditions likely to be encountered in their natural habitat, and at a time in their life when they are highly susceptible to the toxicant tested.

The toxicity of a test medium can change in the course of a test, owing to the gradual escape, decomposition, or detoxification of some constituents, to the accumulation of metabolic products, and to other causes. The ensuing bias of the bioassay result can be serious. The toxicity of a waste determined by the routine bioassay procedure can be less than its actual toxicity in a receiving stream if the potency of the sample declines during the test. In a receiving stream the fish maintain their position and are therefore continually exposed to fresh waste. Results more applicable to situations of this kind can be obtained by the use of modifications of the routine method involving renewal of test solutions, such as continuous-flow bioassay.

10. Reporting and Application of Bioassay Results

10.1. Reporting of experimental data and results: A report of a toxicity bioassay always should include information on the kind of fish used as test animals, their source and average size, the test temperature, and also the source of the experimental water and its composition (mineral content), or at least its more important characteristics, such as the total alkalinity, pH, hardness, any pronounced turbidity or considerable salinity, and the known or possible presence of any unusual natural constituents or contaminants. Other pertinent data, such as the volume and depth of the liquid medium and the number and total weight of fish in each test container, and a summary of the results of chemical determinations made in the course of the tests (particularly the recorded DO and pH values), also can be instructive and helpful.

A complete report should include, in addition to the TL_m values determined, a concise statement or table of the experimental data on which these values are based; that is, the number of fish used and the recorded percentages of survival at each tested concentration.

10.2. Significance and practical application of bioassay results: The TL_m values are useful measures of the acute toxicity of the tested substances under certain experimental conditions, but they obviously do not represent concentrations which may be deemed safe, or harmless, in fish habitats subject to pollution. Concentrations of wastes which are not demonstrably toxic to fish within 96 hr may be very toxic under conditions of continuous exposure in a receiving water. Under stream conditions and with prolonged exposure, toxic levels may be only a small fraction of the determined 48-hr or 96-hr TL_m values. Therefore, when estimating safe discharge rates or dilution ratios for industrial effluents or other pollutants on the basis of acute-toxicity evaluations, one must make use of liberal "safety factors", more properly termed "application factors". Even the provision of an apparently ample margin of safety can fail to accomplish its purpose when there is accumulative toxicity which cannot be predicted from acute-toxicity bioassay results, and when the choice of test animals, of experimental water, or of experimental procedure, or the sampling of wastes for bioassay, has been improper or unfortunate.

No single, simple application factor can be valid

for all wastes or toxicants. The constituents of a complex waste that are responsible for the acute toxicity of the waste may be, but are not necessarily, the constituents responsible for such chronic or cumulative toxicity as may be demonstrable when the waste has been diluted enough so that it is no longer acutely toxic. The chronic toxicity may be lethal after a long time, or it may cause only non-lethal impairment of functions or performance of the animals, such as their ability to swim, their appetite and growth, their resistance to disease, their reproductive capacity, and their ability to compete with other forms in the natural environment. The acute toxicity bioassays cannot be expected to reveal such effects, nor to indicate reliably at what waste concentrations they will or will not occur. Nevertheless, knowledge of the acute toxicity of a waste often can be very helpful in predicting or anticipating and preventing delayed damage to aquatic life in receiving waters, as well as in regulating toxic waste discharges so as to avoid rapid mortality of fish exposed to the toxicants for relatively short periods of time.

Formulas for the estimation of waste concentrations compatible with indefinite survival and well-being of various

fish and other valuable aquatic organisms in receiving waters need to be developed and widely tested. Such formulas have been tentatively proposed and procedures for their derivation and verification have been described or suggested and discussed elsewhere.^{1, 8-10, 12} A review article¹¹ on the water quality requirements of fish can serve as a general introduction to physiologic, toxicologic, and ecologic fundamentals.

MODIFICATIONS OF ROUTINE BIOASSAY METHOD

1. Controlled Artificial Oxygenation of Test Solutions

Artificial oxygenation of test solutions may be employed when excessive biochemical or other oxygen demand of a waste is found to interfere with the evaluation of its toxicity by the routine method. Vigorous aeration with compressed air has not generally proved satisfactory in work with industrial wastes because of rapid losses of toxicity that often result.

A method for controlled oxygenation without accelerating the rate of escape of volatile substances has been described in detail by Doudoroff et al.⁵ This method consists of using a partially closed vessel and carefully regulating the release of bubbles of air and oxygen so that

the required oxygen concentration can be maintained without loss of volatile material in excess of the loss during tests by the routine method. The rate of escape of free carbon dioxide in water is used for calibrating the bubbling rate in the test container. Bubbles of air and oxygen are introduced at the predetermined rate, through glass tubing, near the bottom of the test container.

A similar method of controlled release of oxygen has been used successfully for bioassays with many types of industrial effluents in open test aquaria. In many instances, the addition of pure oxygen in the form of large bubbles at a slow rate (30 to 180 bubbles per minute) to the open test containers used in the routine bioassay method is quite satisfactory for maintaining oxygen concentrations without excessive loss of volatile materials or toxicity. An oxygen cylinder, a pressure reduction valve, necessary tubing, and three-way air valves described by Henderson and Tarzwell⁹ are satisfactory for this purpose. Oxygen can be introduced into test solutions by means of glass tubing (a 5-mm inside diameter has been satisfactory with numerous industrial effluents) and the rate of application adjusted as necessary. Careful regulation is necessary to maintain adequate dissolved

oxygen and avoid any considerable supersaturation.

Another method of oxygenating test solutions in open test jars has been described by Hart, Weston, and De-Mann.³ An interface of adjustable area between the liquid tested and oxygen gas is maintained inside a submerged, inverted funnel with perforations near the base. A small impeller inside the funnel gently agitates the liquid, accelerating the absorption and diffusion of oxygen.

Sometimes it may be satisfactory simply to maintain an oxygen-enriched atmosphere over the still surface of the test solutions in large partially-filled bottles or other suitable test containers.

2. Initial Oxygenation of the Diluent

Another modification of the routine bioassay procedure is the addition of oxygen to the dilution water. An industrial effluent can be free of oxygen, and it also can have a considerable immediate (chemical) oxygen demand, which must be satisfied. The DO content of each test dilution must be adequate at the beginning of the test; however, excessive supersaturation during the test should be avoided. In order to introduce the required amount of oxygen without recourse to artificial aeration or excessive dilution of the

test waste, additional oxygen may be dissolved in the diluent water before it is mixed with the waste. This can be done by bubbling compressed oxygen gas (released from a disperser) through the water in a tall container. The oxygen content or the immediate oxygen demand of the waste sample should be determined. The required oxygen content of the diluent to be added, or the minimum degree of dilution with well-oxygenated (supersaturated) water of known oxygen content that will insure an adequate initial DO level, can then be estimated.

3. Renewal of Test Solutions

A third modification of the routine procedure involves renewal of the liquids tested, for the purpose of maintaining more or less uniform concentrations of any volatile and unstable toxic components and adequate DO content. This modification is recommended especially when there are reasons for believing that the toxicity of the liquids declines rapidly during the course of a test. Rapid reduction of toxicity can result from extreme volatility of important dissolved substances. It can also be due to destruction or removal from solution of toxic constituents by oxidation, hydrolysis, or precipitation, or by their combina-

tion with metabolic products or with mucus of the test animals, accumulation or metabolic destruction within the tissues of the test animals, and so forth. Accumulation of metabolic products sometimes may cause an increase of the toxicity of the liquids tested.

Constant-flow systems, in which the diluted wastes or test solutions are renewed continually (that is, replaced with fresh mixtures flowing constantly into the experimental containers), have proved very useful in research laboratories. With such apparatus, constancy of the composition and oxygen content of the test liquids can be insured. For practical reasons, however, the use of constant-flow apparatus cannot be generally recommended for routine application at this time.

Periodic renewal of the liquid tested, at daily or other convenient intervals, presents fewer difficulties. This can be accomplished by transferring the test animals quickly, by means of a dip net, to a test container with fresh liquid. Renewal of the test medium at intervals of 24 hr is often both convenient and sufficient, but renewal at shorter intervals, such as 12 or 8 hr, sometimes is necessary or advisable. When the oxygen demand of the substance

tested is great, artificial oxygenation of the test solutions may also be needed in order to maintain an adequate DO concentration. This is not often necessary, however, if the liquids are renewed at intervals of 24 hr or less.

4. Tests of Prolonged Duration

The duration of routine tests has been more or less arbitrarily limited to 96 hr. More prolonged tests sometimes are deemed desirable, inasmuch as even acute toxicity does not always cause death within the 96-hr test period. When some test animals are still alive but dying or evidently affected after a 96-hr exposure to some concentrations of a toxic material, the advisability of prolonging the tests is indicated. Median tolerance limits for longer exposure periods (e.g., 10 days) thus can be determined. If tests are continued for periods longer than 10 days, the test fish may have to be fed.

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APPENDIX IV

CHEMICAL OXYGEN DEMAND

Scope

This method is used to determine the chemical oxygen demand (COD) of water and wastewater. The values obtained using this method are sometimes referred to as "oxygen consumed" or "dichromate oxygen demand".

It is important that the same technique be used each time, since only part of the organic matter is oxidized. The proportion oxidized depends on the oxidant used, the structures of the organic compounds present, and the analytical technique. The use of potassium dichromate, described here, has advantages of reproducibility and applicability to a wide variety of effluents.

The method is used to determine COD levels in the range 20-50 ppm. The range can be extended by proper dilution of the sample in the case of high COD levels, or by dilution of the dichromate for low COD levels. Use of this method for COD levels below 20 ppm is not recommended except to indicate orders of magnitude.

Apparatus

- (1) Reflux apparatus, consisting of a hot plate or similar heating unit, a 300 millilitre round-bottom flask with 24/40

ground glass neck, and a Friedrich's condenser.

(2) Powder funnel, approximately 75 millimeters diameter.

Reagents

Deionized, distilled water must be used in the preparation of all reagents.

(a) Standard Potassium Dichromate Solution 0.250N

Dissolve, in distilled water, 12.259 grams $K_2Cr_2O_7$, primary standard or reagent grade, previously dried at $105^{\pm} 2^{\circ}C$ for 2 hours prior to weighing. Dilute to exactly one litre in a volumetric flask.

(b) Sulphuric Acid, Concentrated

Reagent Grade H_2SO_4 (Sp. Gr. 1.84).

(c) Standard Ferrous Ammonium Sulphate Solution 0.25N

Dissolve 98 grams $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Mohr's salt) in distilled water.

Add 20 millilitres conc. H_2SO_4 , cool, and dilute to one litre in a volumetric flask. This solution must be standardized against the standard $K_2Cr_2O_7$ daily, as follows:

Standardization: Dilute 25.0 millilitres standard dichromate solution to about 250 millilitres. Add 20 millilitres of conc. H_2SO_4 and allow to cool.

Titrate with the ferrous ammonium sulphate using

2 or 3 drops of Ferroin indicator.

$$\text{Normality} = \frac{\text{ml. } \text{K}_2\text{Cr}_2\text{O}_7 \times 0.25}{\text{ml. } \text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2}$$

(d) Ferroin Indicator Solution

Dissolve 1.50 grams of 1,10-phenanthroline mono-hydrate, together with 0.70 grams $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in distilled water and dilute to 100 millilitres. (This indicator is commercially available, requiring no preparation.)

(e) Mercuric Sulphate, HgSO_4

Reagent grade.

(f) Silver Sulphate, Ag_2SO_4

Reagent grade.

Procedure

The principle involved in this test procedure is that the carbonaceous (organic) material in the sample is oxidized by a known amount of potassium dichromate, and the residual dichromate is determined by titration with standard ferrous ammonium sulphate using Ferroin indicator. Mercuric sulphate and silver sulphate are used as catalysts in the oxidation to ensure complete reaction and also to avoid error due to chlorides. A sample of deionized, distilled

water treated under identical conditions is used as a blank.

Place a 50 millilitre sample, or an aliquot diluted to 50 millilitres with deionized, distilled water, in the round bottom flask and add 25 millilitres standard dichromate solution. Add boiling stones to prevent bumping (8-10 mesh Carborundum is recommended).

Using a powder funnel add 1.0 gram of mercuric sulphate and 1.0 gram of silver sulphate to the flask. The neck of the funnel must be long enough to extend below the neck of the boiling flask.

Carefully add, in small aliquots or in a slow steady stream with continuous agitation of the flask contents, 75 millilitres concentrated H_2SO_4 . Continue to agitate the contents of the flask for at least one minute to ensure complete mixing of all components.

The reflux mixture must be thoroughly mixed before heat is applied. If this is not done, local heating occurs in the bottom of the flask and the mixture may be blown out of the side arm of the condenser.

Attach the flask to the Friedrich's condenser (do not use stopcock grease). Turn on the hot plate and the water to the condenser. Bring the contents of the flask to

a boil and reflux for 2 hours. Cool, and wash down the condenser with about 25 millilitres deionized, distilled water.

Transfer the contents of the flask to a 500 millilitre Erlenmeyer flask and rinse out the reflux flask 4-5 times with deionized, distilled water, adding the rinsings to the Erlenmeyer flask.

Dilute the mixture to about 350 millilitres with deionized, distilled water and cool to room temperature.

Add 2-3 drops Ferroin indicator and titrate the excess dichromate with the freshly standardized 0.25N ferrous ammonium sulphate.

A blank, consisting of 50 millilitres deionized, distilled water instead of the sample, is refluxed under conditions identical to those described above including the addition of identical volumes of rinse and dilution water.

Titrate the blank sample with 0.25N ferrous ammonium sulphate using the same amount of indicator as used for the test sample. It is important that the same amount of indicator be used in the blank as in the sample, since this titration also constitutes an indicator blank.

Calculations

Calculate the COD content as follows:

$$\text{ppm COD} = \frac{(A-B) \times C \times 8,000}{\text{millilitres sample}}$$

where A = millilitres ferrous ammonium sulphate
used for titration of blank.

B = millilitres ferrous ammonium sulphate
used for titration of sample.

C = normality of ferrous ammonium sulphate
as standardized against standard dichromate
solution.

Using this method, it is unnecessary to correct
for the chloride content unless extremely high accuracy is
required. The correction, if required, is calculated as
follows:

$$\text{ppm Chloride Correction} = \text{ppm Chloride} \times 0.007 \times \text{reflux time (hours)}$$

The complete calculation for COD thus becomes:

$$\text{ppm COD} = \frac{(A-B) \times C \times 8,000}{\text{millilitres sample}} - \text{Chloride Correction (ppm)}$$

NOTE 1

If it is desired to determine the COD due to dis-

solved solids only, the filtrate from the ten mesh screen should be filtered again through a filter paper equivalent to a Reeve Angel No. 202. The filtrate should then be analyzed as in the given procedure.

In the case of filtrates with very low COD (e.g., raw water), dust from the filter paper should be washed away with 500 millilitres of deionized, distilled water before filtering the sample.

Additional Information

- (1) The amount of indicator is left to the operator's discretion, but should not vary substantially from the indicated amount.
- (2) The endpoint goes from a blue-green to gray to reddish-brown. The endpoint will not be as sharp as in the standardization of the reagents because of the higher acid concentration in the sample. For this reason, it is necessary that the sample be diluted to at least 350 millilitres before the titration is carried out.
- (3) The presence of minute quantities of organic material in the distilled water can affect the results. For extremely accurate results, water distilled from a solution of potassium chromate and sulphuric acid should be used throughout.

(4) For samples with a very low COD, the dichromate solution is diluted to 0.025N. Extreme care must be taken with all glassware at this concentration because the slightest trace of organic matter in the condenser, flask, or atmosphere will cause gross errors. Only those samples with approximately 50 percent reduction of the dichromate will be reasonably accurate. A dilute ferrous ammonium sulphate solution, 0.025N, should be used for the back titration. This concentration must be prepared using excess acid, or diluted fresh daily from the 0.25N solution.

Bibliography

- (1) "Standard Methods for the Examination of Water and Wastewater", Eleventh Edition, pp. 399-402, American Public Health Association, N.Y.
- (2) Richard A. Dobbs and Robert T. Williams, Analytical Chemistry, Vol. 35, No. 8, pp. 1064-1067.
- (3) James M. Cripps and David Jenkins, Journal of the Water Pollution Control Federation, Vol. 36, No. 10, pp. 1240-1246.

APPENDIX V

ONTARIO MINISTRY OF THE ENVIRONMENT

GUIDELINES FOR THE USE OF CHEMICAL DISPERSANTS FOR OIL SPILLS

The chemical treatment of oil on water by the use of detergents or dispersants as they will be referred to in these guidelines has been the subject of much controversy since their massive use during the Torrey Canyon oil spill in 1967. Because the subsequent research studies into the effects of dispersants after the Torrey Canyon spill have shown that dispersants caused severe adverse effects on the aquatic life in the area, there has developed an increasing awareness on the part of regulatory agencies to limit the use of dispersants for the dispersion of oil on water. Unfortunately, no clear dividing line can be established to define the circumstances under which dispersants can be used and, therefore, it becomes necessary to set forth some general guidelines for their use. Where possible, the Ontario Ministry of the Environment should be consulted* before a dispersant is used, but it is recognized that the emergency nature of many oil spills necessitates an immediate decision on the part of those responsible to effect clean-up. The following guidelines are presented to

ensure that all the pertinent factors are taken into consideration before a decision is made regarding the use of dispersant chemicals.

I Dispersants should not be used where the prime concern is:

- (i) protection of surface water supplies
- (ii) protection of fish spawning areas
- (iii) protection of fishing area
- (iv) protection of beaches
- (v) protection of known waterfowl nesting areas

II Dispersants may be considered for use:

- (i) where a fire and safety hazard is present
- (ii) where the floating oil presents an immediate hazard to local waterfowl
- (iii) for control and clean-up of small oil slicks, and then only after all other possible methods for control, containment and removal of the floating oil have been ruled out.

III Where a high potential for small oil spills exists, the first line of defence should be a mechanical boom. However, the storage of dispersants for emergency use will be permitted. Such use, however, should only be after consideration

of the foregoing points.

IV Where possible, the application of dispersants should be under the supervision of MOE personnel*. In any event, the MOE should be contacted whenever a dispersant is used for an oil spill.

V Pending the publication of the list of oil spill treating agents considered acceptable for use on surface waters in Ontario, dispersants exhibiting the minimum toxicity to aquatic life should be used. It is recognized that a reduced toxicity often means a lower efficiency of the dispersant. The MOE Research Branch is continually studying the toxicity and effectiveness of dispersants, and can be consulted for information on specific proprietary chemicals.

VI When the list of acceptable oil spill treating agents is published, only those OSTAs on this list shall be used for clean-up of oil spills in Ontario.

VII Chemicals have been developed for aiding the combustion of oil, creating chemical booms, gelling of oil, etc., but in most cases, their applicability for use on oil spills is more limited. These chemicals, while they are generally less toxic than dispersants, should not be used without prior consultation with the Ontario Ministry of the Environment,

since other factors must be considered.

* See MOE contact list attached.

MOE Contact List

Re

Use of Chemical Dispersants for Oil Spills

	<u>Office</u>	<u>Home</u>
Toronto - MOE - Ontario Operations Centre	(416) 965-2537 (24-hour service)	

MOE - Industrial Wastes Branch

Mr. R. M. Gotts	(416) 965-6346	(416) 751-0714
Mr. R. C. Stewart	(416) 965-6346	(416) 451-8226
Mr. T. D. Armstrong	(416) 965-6346	(416) 487-0673
Mr. J. W. Vogt	(416) 965-6346	(416) 221-2976
Mr. W. L. Dick	(416) 965-6975	(416) 630-7701

MOE - Research Branch

Mr. A. Oda	(416) 248-3044	(416) 742-4038
London Office	Mr. R. W. Hussain	(519) 433-3961
	Mr. J. Bray	(519) 433-3961
Sudbury Office	Mr. L. W. Fitz	(705) 674-3151
Kingston Office	Mr. L. South	(613) 546-3171
	Mr. G. Macey	(613) 546-3171
Thunder Bay Office	Mr. I. Ramsay	(807) 623-5591
	Mr. J. Marsh	(807) 623-5591
Sarnia Office	Mr. J. Luyt	(519) 336-0130
		(519) 542-6544

APPENDIX VI

THRESHOLD ODOUR TEST

The Threshold Odour Test is primarily directed towards the evaluation of the potability of public water supplies. When applied to effluents and other wastes, the test is indicative of the potential for the effluent or waste to adversely affect the taste and odour of public water supplies and to taint the flesh of fish.

Cause of Odours

Presence of foreign substances, usually organic. Some inorganics like hydrogen sulphide also cause odour.

Source of Odours

The contaminating material may be of natural origin such as algae, dead fish, or may come from domestic or industrial waste discharges.

Purpose of Test

- (1) A check on the quality of the raw and finished water.
- (2) For control of odour through the plant and determination of treatment dosages.
- (3) To check the effectiveness of different kinds of treatment.
- (4) A means of tracing the source of contamination.
- (5) A written record on the condition of the water leaving

the plant which may be very useful in dealing with complaints.

Definition of Threshold Odour

The "Threshold Odour Number" represents the extent to which an odour-bearing water must be diluted with odour-free water to reduce the odour to a concentration which is just perceptible.

Sensitivity

It is possible to differentiate between odour concentrations only 15% apart.

Interference

(a) If the members of the panel are consistently exposed to a particular odour they may become insensitive to its presence. For example, the presence of chlorinous odours in a water is particularly difficult for a plant operator to detect because he is almost continuously subjected to the odour of chlorine around the treatment plant.

(b) The test should be run in a room devoid of foreign odours. Odours caused by fresh paint, volatile solvents, tobacco smoke, food, etc., will decrease the accuracy of the test. The panel member's hands should, therefore, also be rinsed free of soap, tobacco or food odours.

(c) Wait at least an hour after meals before running the test. Avoid imbibing alcoholic beverages as they have a particularly adverse effect.

Selecting the Panel

The panel is supposed to be representative of the average consumer, and thus it is undesirable to restrict membership on the panel to those with an extremely sensitive sense of smell. On the other hand, completely insensitive persons should not be used.

A word of warning. Panel members should never declare a dilution positive unless there is sufficient odour present to clearly identify it. Failure to observe this precaution can result in guessing which leads to incorrect results.

Apparatus

- (1) Six 500 ml erlenmeyer flasks with ground glass stoppers.
- (2) One thermometer (0 - 110° C).
- (3) One 250 ml graduated cylinder.
- (4) One 100 ml graduated cylinder.
- (5) One 10 ml graduated pipette.
- (6) One large hot plate (preferably electric) or water bath.
- (7) One odour-free water generator.

(8) Two large flasks for heating odour-free water.

Procedure

(1) Acid wash all the glassware to be used in the test and rinse with odour-free water.

(2) Determine the approximate range of odour value. The experienced operator can usually do this by sniffing the undiluted sample at 60°C ($\pm 2^\circ\text{C}$).

(3) Prepare a series of dilutions covering the anticipated range. With odour-free water, bring the volume in all the flasks to 200 ml.

(4) Place the flasks on the hot plate, including a blank containing 200 ml of odour-free water, and heat to 60°C ($\pm 2^\circ\text{C}$).

(5) Smell the blank, then the sample, and record the result as positive or negative.

(6) The dilution containing the smallest volume of sample in which an odour was detected determines the threshold odour concentration. The threshold number is calculated by dividing 200 by the volume of the test sample used for the threshold dilution.

(7) Sometimes samples contain a large amount of chlorine which masks the odourous substance present in the water.

The chlorine may be destroyed by adding some sodium thiosulphate to the water. The absence of chlorine from the sample may be determined by the orthotolidine test.

TABLE I

Threshold Odour Numbers Corresponding to
Various Dilutions Temperature 60°C

Sample Volume Diluted to 200 ml. ml.	Threshold Odour Number	Sample Volume Diluted to 200 ml. ml.	Threshold Odour Number
200	1	12	17
140	1.4	8.3	24
100	2	5.7	35
70	3	4	50
50	4	2.8	70
35	6	2	100
25	8	1.4	140
17	12	1.0	200

APPENDIX VII

PHENOLS DETERMINATION

Methods for the determination of phenols are detailed in "Standard Methods for the Examination of Water and Wastewater". The most recently revised directions are contained in the 11th Edition. Modifications of these methods have been found satisfactory for many samples in the MOE Laboratories. It is suggested that analysts at other laboratories who may have to deal mainly with samples more subject to interferences may find the routine use of Standard Methods procedures more suitable. In any case, before a method is chosen, the sections in Standard Methods on phenols, and in particular, the prefaces, should be reviewed (Water: page 197, Industrial Wastes: page 403-414).

The majority of samples examined in the MOE chemical laboratory are "clean" samples, samples of raw and treated water supplies, samples obtained throughout the open waters of the Great Lakes and other large bodies of water, and other samples in which the likelihood of interference is remote. Consequently, the Gibbs procedure with some modifications is favoured for these kinds of sample. However, for the purposes of determining phenolics in more complex and concentrated samples, the 4-Amino-Antipyrine procedure with

screening modifications described in Standard Methods is recommended.

IMPROVED 4-AMINO-ANTIPYRENE METHOD

FOR DETERMINATION OF PHENOL

The most reliable method of the three methods suggested in Standard Methods is the 4 A.A.P. Visual comparison (Gibbs Method) can only be used to give an approximate value.

Preservation and Storage of the Sample

The analysis on the sample should be done within four hours of its collection, otherwise the sample should be preserved with 1 gm. per litre of 0.1% of copper sulphate solution. Even after this treatment, the sample should be stored in the dark at a temperature below 20° C.

Oxidizing agents such as chlorine should be eliminated by the addition of sodium arsenite. If the oxidizing agents are not removed in the sample, the phenolic compounds will partially oxidize and the results obtained will be low.

To overcome other interferences, the sample should be distilled. To 100 mls. of the preserved sample, add 2 or 3 drops of methyl red and acidify with 3 mls. of 15% phosphoric acid. If sulphide is detected, add lead carbonate to precipitate the sulphide. Distill and collect 90 mls. in

the graduate. Stop distillation, let cool, add 10 mls. of distilled water and continue distillation until 100 mls. are collected. This distillate is used for analysis by the 4 A.A.P. method.

Steam volatile phenols react with 4-amino-antipyrine at a pH of 10.0 ± 0.2 in presence of potassium ferricyanide to form a coloured anti-pyrene dye. This dye is extracted from aqueous solution with chloroform and the absorbance is measured at 460 μ m on a spectrophotometer.

Reagents

The reagent strength has been slightly modified to give better sensitivity. All reagents should be prepared with boiled and cooled distilled water (phenol free).

Buffer Solution

Dissolved 33.2 gms. ammonium chloride in 127 mls. ammonia solution ($d = 0.880$) and dilute to 250 mls. with distilled water.

4-Amino-Antipyrine Solution

Dissolved 0.25 gms. 4 A.A.P. in distilled water and dilute to 25 mls. Prepare fresh each day.

Potassium Ferricyanide

Dissolved 8 gms. potassium ferricyanide in distilled

water and dilute to 100 mls. Filter if necessary. Prepare fresh each week.

Chloroform - Reagent grade

Sodium Sulphate - Anhydrous granular

Stock Phenol Solution

Dissolve 1 gm. reagent grade phenol in freshly boiled and cooled distilled water and dilute to 100 mls.

1 ml. stock = 1 mg. phenol

Intermediate Phenol Solution

Dilute 10.0 mls. stock phenol solution to 1000 mls. with freshly boiled and cooled distilled water. Prepare fresh each day.

1 ml. intermediate solution = 10 ug. phenol

Standard Phenol Solution

Dilute 50 mls. intermediate phenol solution to 500 mls. with freshly boiled and cooled distilled water. Prepare this solution within 2 hours of use.

1 ml. standard = 1 ug phenol

Preparation of Calibration Graph

Into each of a series of 1 litre separatory funnel, add volumes of the standard solution corresponding to 0, 2, 5, 10, 15, 20, 25 ug. phenol and dilute to 500 mls. with

distilled water. Treat each standard as follows:

First the pH should be set at 10.0 ± 0.2 . Add 2 mls. buffer solution, mix well. Add 1 ml. 4 A.A.P. solution, mix well. Add 5 mls. potassium ferricyanide solution again, mix well, and allow the colour to develop for 15 minutes. The pH of the aqueous layers should be 10.0 ± 0.2 . The solution should be clear and light yellow. Extract immediately with 25 mls. chloroform. Shake each 10 times and allow the chloroform to settle again. Run each chloroform extract into a dry 50 mls. volumetric flask containing about 2 gms. sodium sulphate in the filter funnel. Do not add more chloroform but replace stopper and swirl the contents of the flask gently. Fill 4 cm. glass cell with extract. Measure the absorbance of the extract against distilled water at 460 mu. Correct each absorbance for the blank reading and plot against ug phenol to obtain the calibration graph.

Analysis of the Sample

Place 500 mls. of distillate in a 1 litre separatory funnel and proceed as given under "Preparation of Calibration Graph" starting with 2 mls. buffer solution (usually more than 2 mls. buffer is needed to get a pH of 10.0 ± 0.2). Check the pH after adding the buffer.

Prepare one standard with each series of analyses as a check on the calibration graph.

Measure the absorbance of the sample extract and blank in 4 cm. cells against distilled water at a 460 mu. Deduct the reading obtained for the blank and read off the phenol content in ug in the sample from the calibration curve.

Calculation: ppb phenol = ug phenol from calibration curve x 1,000
original sample aliquot in mls.

NOTES

PHENOLS - GIBBS PROCEDURE

PHENOLS - 4 A.A.P. PROCEDURE

(1) At the Ontario Ministry of the Environment laboratory, phenols are recovered from samples by one of three methods:

- (a) straight-run (i.e., "as received")
- (b) screening
- (c) distillation

Analysis is done by one of two methods:

- (1) Gibbs method
- (2) 4-Amino-Antipyrene method

Either analysis may be couple with any one of the three recovery methods giving rise to diversification of laboratory procedure.

(2) The Gibbs method recovers ortho and meta phenols along with quantities of para phenols. However, some p-phenols are not detected (i.e., p-cresol). Therefore, the answer recorded by the analyst represents the minimum concentration of phenols present in the original sample rather than the maximum.

(3) the 4 A.A.P. method for the determination of phenol concentrations probably does not recover all the p-phenols.

This is because most of the samples (like the samples for the Gibbs test) must suffer a primary distillation. Therefore, the losses for both tests may be somewhat similar.

(4) Phenol losses apparently occur during screening, distilling and at just about every stage in the procedures.

(5) A 100 ml. sample is required for "straight run" analysis (i.e., clean, clear, river water, drinking water, etc.). If a sample has been preserved, the entire bottle will be used (or a suitable aliquot will be withdrawn and the rest of the sample will be discarded). After preservation, a sample is "hybrid" - that is, not representative of the original sample source. It cannot be used for further tests.

(6) The minimum concentration of phenols that can be detected by the Gibbs method (visual - comparative) is 2 parts per billion.

(7) The Gibbs method is inaccurate when high concentrations of phenols (1,000 ppb) are present in the sample. The 4 A.A.P. method (colorimetric) is accurate in the high ranges of phenol concentrations but is inaccurate in the low concentration ranges. Concentrations below 50 ppb cannot even be picked up by the colorimeter.

(8) Although the 4 A.A.P. procedure can be used to check up

on any high values obtained by the Gibbs method, it is not used extensively in MOE laboratories.

(9) Samples to be analyzed for phenolic content should be preserved and stored unless analysis is to be accomplished within 4 hours after collection.

(10) The following are methods of preserving aqueous phenolic samples:

For best preservation, use all four steps in the order indicated:

(1) acidify to a pH of approximately 4.0 with H_3PO_4 using methyl orange indicator paper or a pH meter.

(2) if H_2S or SO_2 are known to be present, briefly aerate or stir the sample with caution.

(3) biochemical oxidation of phenols is inhibited by the addition of 1.0 gms. (a "pinch") of $CuSO_4 \cdot 5H_2O$ per litre of sample (or 2 - 3 mls. 10% $CuSO_4$ solution per 100 mls. sample).

(4) the sample should be refrigerated (5 - 10^oC).

Preserved and stored samples should be analyzed within 24 hours of collection.

(11) Do not collect phenol samples in polyethylene bottles (contamination).

(12) Adequate laboratory controls should ensure that outside phenol contamination is eliminated.

APPENDIX VIII

BIOCHEMICAL OXYGEN DEMAND

The oxygen demand of sewage, sewage plant effluents, polluted waters, or industrial wastes is exerted by three classes of materials: (a) carbonaceous organic material usable as a source of food by aerobic organisms; (b) oxidizable nitrogen derived from nitrite, ammonia, and organic nitrogen compounds which serve as food for specific bacteria (e.g., *Nitrosomonas* and *Nitrobacter*); and (c) certain chemical reducing compounds (ferrous iron, sulphite, and sulphide) which will react with molecularly dissolved oxygen. In raw and settled domestic sewage, most--and, for practical purposes, all--of the oxygen demand is due to the first class of materials and is determined by the biochemical oxygen demand (BOD) test described below. In biologically treated effluents, a considerable proportion of the oxygen demand may be due to oxidation of Class (b) compounds and will also be included in the BOD test. Class (c) materials present may not be included in the BOD test unless the test is based on a calculated initial dissolved oxygen. It should be understood that all three of these classes will have a direct bearing on the oxygen balance of the receiving water and must be considered in the discharge of a waste to such a water.

If wastes consisted only of raw or treated domestic sewage, measurement of the oxygen load on a receiving water would be simple. Unfortunately, this is not always the case, because most wastes are complex in nature and may contain organic compounds not easily amenable to biologic oxidation. When such compounds are present, the usual methods of seeding and the standard incubation period of 5 days will fail to assess the effect these wastes may have at some point below their point of discharge.

Complete stabilization of a given waste may require a period of incubation too long for practical purposes. For this reason, the 5-day period has been accepted as standard. For certain industrial wastes, however, it may be advisable to determine the oxidation curve obtained. Conversion of data from one incubation period to another can only be made if such special studies are carried out. Studies in recent years have shown that the exponential rate of carbonaceous oxidation, k , at 20°C rarely has a value of 0.1, but may vary from less than one-half to more than twice this value. This fact usually makes it impossible to calculate the ultimate carbonaceous demand, L , of a sample from 5-day BOD values unless the k value has been determined on the sewage, waste,

or stream under consideration.

(1) Apparatus

1.1. Incubation bottles, 250-300-ml capacity, with ground-glass stoppers. Bottles should be cleaned with a good detergent and thoroughly rinsed and drained before use. As a precaution against drawing air into the dilution bottle during incubation, a water seal is recommended. Satisfactory water seals are obtained by inverting the bottles in a water bath or adding water to the flared mouth of special BOD bottles.

1.2. Air incubator or water bath, thermostatically controlled at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$. All light should be excluded to prevent formation of DO by algae in the sample.

(2) Reagents

2.1. Distilled water: Water used for solutions and for preparation of the dilution water must be of the highest quality, distilled from a black tin or all-glass still, contain less than 0.01 mg/l copper, and be free of chlorine, chloramines, caustic alkalinity, organic material, or acids.

2.2. Phosphate buffer solution: Dissolve 8.5 g potassium dihydrogen phosphate, KH_2PO_4 , 21.75 g dipotassium hydrogen phosphate heptahydrate, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 1.7 g ammonium

chloride, NH_4Cl , in about 500 ml distilled water and dilute to 1 litre. The pH of this buffer should be 7.2 without further adjustment. If dilution water is to be stored in the incubator, the phosphate buffer should be added just prior to using the dilution water.

2.3. Magnesium sulphate solution: Dissolve 22.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in distilled water and dilute to 1 litre.

2.4. Calcium chloride solution: Dissolve 27.5 g anhydrous CaCl_2 in distilled water and dilute to 1 litre.

2.5. Ferric chloride solution: Dissolve 0.25 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in distilled water and dilute to 1 litre.

2.6. Acid and alkali solutions, 1N. For neutralization of waste samples which are either caustic or acidic.

2.7. Sodium sulphite solution, 0.025N. Dissolve 1.575 g anhydrous Na_2SO_3 in 1,000 ml distilled water. This solution is not stable and should be prepared daily.

2.8. Seeding material: The selection of the proper seed is an important factor in the BOD determination. In many cases, particularly in food-processing wastes, a satisfactory seed may be obtained by using the supernatant liquor from domestic sewage which has been stored at 20°C for 24-36 hours.

Many industrial wastes contain organic compounds which are not amenable to oxidation by domestic-sewage seed. In these cases, the analyst may use seed prepared from soil, acclimated seed developed in the laboratory, or the receiving water collected below the point of discharge of the particular waste (preferably 2-5 miles below). The last two are the most likely possibilities. Receiving water used as a seed source will undoubtedly give the best estimate of the effect of a waste on such a water; but it must be collected at a point where there has been built up a biota capable of using for food the particular organic compounds present. In some cases, this might entail the collection of a satisfactory seed many miles below the point of discharge of the waste, which might not be practical. With recurrent wastes not easily susceptible to biologic oxidation, it is usually more practical to build up an acclimated seed in the laboratory. This may be done by aerating and feeding sewage or receiving water with small daily increments of the particular waste, together with sewage, until a satisfactory seed is developed.

(3) Procedure

3.1. Preparation of dilution water: The distilled water used should have been stored in cotton-plugged bottles

for a sufficient length of time to become saturated with DO. The water may also be aerated by shaking a partially filled bottle or with a supply of clean compressed air. Situations may be encountered where it is desired to use stabilized river water to check stream performance with laboratory procedure. The distilled water used should be as near 20°C as possible and of the highest purity. Place the desired volume of distilled water in a suitable bottle and add 1 ml each of phosphate buffer, magnesium sulphate, calcium chloride, and ferric chloride solutions for each litre of water.

3.2. Seeding: If necessary, the dilution water is seeded by using the seed found to be the most satisfactory for the particular waste under study. Only past experience can determine the actual amount of seed to be added per litre. Seeded dilution water should be used the same day it is made.

3.3. Pretreatment:

(2) Samples containing caustic alkalinity or acidity: Neutralize to about pH 7.0 with 1N H_2SO_4 or NaOH, using a pH meter or bromthymol blue as an outside indicator. The pH of the seeded dilution water should not be changed by the preparation of the lowest dilution of sample.

(b) Samples containing residual chlorine compounds: If the samples are allowed to stand for 1 to 2 hours, the residual chlorine will often be dissipated. BOD dilutions can then be prepared with properly seeded standard dilution water. Higher chlorine residuals in neutralized samples should be destroyed by adding sodium sulphite. The appropriate quantity of sodium sulphite solution is determined on a 100-1,000-ml portion of the sample by adding 10 ml of 1 + 1 acetic acid or 1 + 50 H_2SO_4 followed by 10 ml potassium iodide solution (10 g in 100 ml) and titrating with 0.025N sodium sulphite solution to the starch-iodide endpoint. Add to a volume of sample the quantity of sodium sulphite solution determined by the above test, mix, and after 10-20 min test aliquot samples for residual chlorine to check the treatment. Prepare BOD dilutions with seeded standard dilution water.

(c) Samples containing other toxic substances: Samples such as those from industrial wastes frequently require special study and treatment--for example, toxic metals derived from plating wastes.

(d) Samples supersaturated with DO: Samples containing more than 9.17 mg/l DO at 20°C may be encountered during winter

months or in localities where algae are actively growing.

To prevent loss of oxygen during incubation of these samples, the DO should be reduced to saturation by bringing the sample to about 20°^oC in a partly filled bottle and agitating it by vigorous shaking or by aerating with compressed air.

3.4. Dilution technique: Make several dilutions of the prepared sample so as to obtain the required depletions. The following dilutions are suggested: 0.1-1.0 percent for strong trade wastes, 1-5 percent for raw and settled sewage, 5-25 percent for oxidized effluents, and 25-100 percent for polluted river waters.

(a) Carefully siphon standard dilution water, seeded if necessary, into a graduated cylinder of 1,000-2,000-ml capacity, filling the cylinder half full without entrainment of air. Add the quantity of carefully mixed sample to make the desired dilution and dilute to the appropriate level with dilution water. Mix well with a plunger-type mixing rod, avoiding entrainment of air. Siphon the mixed dilution into two BOD bottles, one for incubation and the other for determination of the initial DO in the mixture; stopper tightly and incubate for 5 days at 20°^oC. The BOD bottles should be water sealed by inversion in a tray of water in the incubator

or by using a special water-seal bottle. Prepare succeeding dilutions of lower concentration in the same manner, or by adding dilution water to the unused portion of the preceding dilution.

(b) The dilution technique may be greatly simplified when suitable amounts of sample are measured directly into bottles of known capacity with a large-tip volumetric pipet and the bottle is filled with just sufficient dilution water so that the stopper can be inserted without leaving air bubbles. Dilutions greater than 1:100 should be made by diluting the waste in a volumetric flask before it is added to the incubation bottles for final dilution.

3.5. Determination of DO: If the sample represents 1 percent or more of the lowest BOD dilution, determine DO on the undiluted sample. This determination is usually omitted on sewage and settled effluents known to have a DO content of practically zero. With samples having an immediate oxygen demand, a calculated initial DO should be used, inasmuch as such a demand represents a load on the receiving water.

3.6. Incubation: Incubate the blank dilution water and the diluted samples for 5 days at 20°C. Then determine the

DO in the incubated samples and the blank, using the azide modification of the iodometric method (A). In special cases, other modifications may be necessary. Those dilutions showing a residual DO of at least 1 mg/l and a depletion of at least 2 mg/l should be considered the most reliable.

3.7. Seed correction: If the dilution water is seeded, determine the oxygen depletion of the seed by setting up a separate series of seed dilutions and selecting those resulting in 40-70 percent oxygen depletions in 5 days. One of these depletions is then used to calculate the correction due to the small amount of seed in the dilution water. Do not use the seeded blank for seed correction because the 5-day seeded dilution water blank is subject to erratic oxidation due to the very high dilution of seed, which is not characteristic of the seeded sample.

3.8. Dilution water control: Fill two BOD bottles with unseeded dilution water. Stopper and water-seal one of these for incubation. The other bottle is for determining the DO before incubation. The DO results on these two bottles are used as a rough check on the quality of the unseeded dilution water. The depletion obtained should not be used as a blank correction; it should not be more than 0.2 ml and

preferably not more than 0.1 ml.

3.9. Glucose-glutamic acid check: The BOD test is a bioassay procedure; consequently, the results obtained are influenced greatly by the presence of toxic substances or the use of a poor seeding material. Experience has taught that distilled waters are frequently contaminated with toxic substances--most often copper--and that some sewage seeds are relatively inactive. The results obtained with such waters are always low.

The quality of the dilution water, the effectiveness of the seed, and the technique of the analyst should be checked periodically by using pure organic compounds on which the BOD is known or determinable. If a particular organic compound is known to be present in a given waste, it may well serve as a control on the seed used. There have been a number of organic compounds proposed, such as glucose or glutamic acid. For general BOD work, a mixture of these (150 mg/l of each) has certain advantages. It must be understood that glucose has an exceptionally high and variable oxidation rate with relatively simple seeds. When used with glutamic acid, the oxidation rate is stabilized and is similar to that obtained with many municipal wastes (0.16-

0.19 exponential rate). In exceptional cases, a given component of a particular waste may be the best choice to test the efficacy of a particular seed.

The standard glucose solution should show a BOD of 224 ± 10 mg/l; the standard glutamic acid solution should show a BOD of 217 ± 10 mg/l. Any appreciable divergence from these results raises a serious question concerning the quality of the distilled water or the viability of the seeding material. Further, if a variation greater than $\pm 20-22$ mg/l occurs more frequently than 5 percent of the time, the technique used is subject to improvement.

To check the dilution water, the seed material, and the technique of the analyst, prepare a standard solution containing 150 mg/l each of reagent grade glucose and glutamic acid which have been dried at 103°C for 1 hour. Pipet 5.0 ml of this solution into calibrated incubation bottles, fill with seeded dilution water, and incubate with seed control at 20°C for 5 days. On the basis of a mixed primary standard containing 150 mg/l each of glucose and glutamic acid, the 5-day BOD varies in magnitude according to the type of seed, and precision varies with the quality of seed, as shown in Table I.

Excepting the oxidized river water and effluents, a low seed correction resulted in an appreciably higher value for the standard deviation. Each seed source should be checked to determine the amount required to obtain optimum precision. If results differ appreciably from those given in Table I after considering the seed source, the technique is questionable.

TABLE I
EFFECT OF SEED TYPE AND QUALITY ON BOD RESULTS

<u>Type of Seed</u>	<u>5-Day Seed Correction mg/l</u>	<u>Mean 5-Day BOD mg/l</u>	<u>Standard Deviation mg/l</u>
Settled fresh sewage	> 0.6	218	\pm 11
Settled stale sewage	> 0.6	207	\pm 8
River water (4 sources)	0.05-0.22	224-242	\pm 7-13
Activated-sludge effluent	0.07-0.68	221	\pm 13
Trickling filter effluent	0.2-0.4	225	\pm 8

(4) Immediate Dissolved Oxygen Demand

Substances oxidizable by molecular oxygen, such as ferrous iron, sulphite, sulphide, and aldehyde, impose a load on the receiving water and must be taken into consideration. The total oxygen demand of such a substrate may be determined by using a calculated initial DO or by using the sum of the immediate dissolved oxygen demand (IDOD) and the

5-day BOD. Where a differentiation of the two components is desired, the IDOD should be determined. It should be understood that the IDOD does not necessarily represent the immediate oxidation by molecular DO, but may represent an oxidation by the iodine liberated in the acidification step of the iodometric method.

The depletion of DO in a standard water dilution of the sample in 15 min has been arbitrarily selected as the IDOD. To determine the IDOD, the DO of the sample (which in most cases is zero) and the DO of the dilution water are determined separately. An appropriate dilution of the sample and dilution water is prepared, and the DO is determined after 15 min. The calculated DO of the sample dilution minus the observed DO after 15 min is the IDOD (mg/l) of the sample dilution.

(5) Calculation

5.1. Definitions:

D_o = DO of original dilution water;

D_1 = DO of diluted sample 15 min after preparation;

D_2 = DO of diluted sample after incubation;

S = DO of original undiluted sample;

D_e = DO available in dilution at zero time = $D_o p + SP$;

p = decimal fraction of dilution water used;
 P = decimal fraction of sample used;
 B_1 = DO of dilution of seed control before incubation;
 B_2 = DO of dilution of seed control after incubation;
 f = ratio of seed in sample to seed in control
= $\frac{\% \text{ seed in } D_1}{\% \text{ seed in } B_1}$; and
Seed correction = $(B_1 - B_2)f$.

5.2. Biochemical oxygen demand:

(a) When seeding is not required:

$$\text{mg/l BOD} = \frac{D_1 - D_2}{P}$$

(b) When using seeded dilution water:

$$\text{mg/l BOD} = \frac{(D_1 - D_2) - (B_1 - B_2)f}{P}$$

(c) Including IDOD if small or not determined:

$$\text{mg/l BOD} = \frac{D_e - D_1}{P}$$

5.3. Immediate dissolved oxygen demand:

$$\text{mg/l BOD} = \frac{D_e - D_1}{P}$$

The DO determined on the unseeded dilution water after incubation is not used in the BOD calculations because this practice would overcorrect for the dilution water. In

all of the above calculations, corrections are not made for small losses of DO in the dilution water during incubation. If the dilution water is unsatisfactory, proper corrections are difficult and the results are questionable.

(6) Precision and Accuracy

There is no standard against which the accuracy of the BOD test can be measured. The precision of the DO determinations in the BOD test, expressed as the standard deviation in millilitres of 0.025N thiosulphate, is as follows:

DO in dilution water: 0.043 ml (n = 3; 6 x 10).

DO in sewage: 0.058 ml (n = 2; 26 x 10).

Incubated BOD samples, 1-20-day BOD (curve),
glucose and glutamic acid: 0.079 ml (n = 1; 27 x 10).

Five-day BOD--

Raw sewage: 0.112 ml (n = 3; 38 x 10)

Settled sewage: 0.071 ml (n = 1; 20 x 5)

Activated sludge effluent: 0.084 ml (n = 3;
30 x 10)

Trickling filter effluent: 0.075 ml (n = 1;
16 x 5)

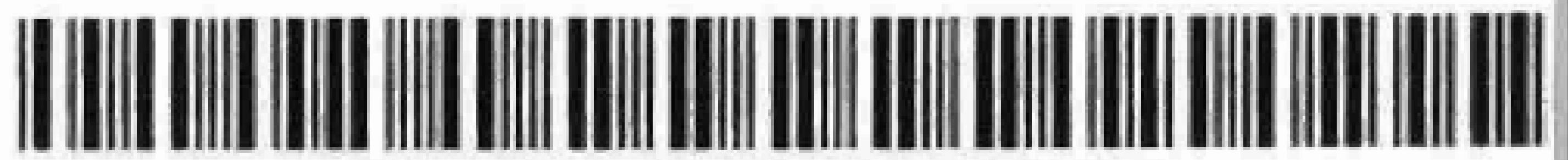
These data indicate that, where interfering and toxic substances are absent, the standard deviation of the BOD test on sewages and effluents may range from 0.07 to 0.11 ml oxygen demand titrated. This should also hold true on wastes from food-processing industries or other organic non-toxic wastes.

Industrial wastes containing toxic materials or substances which interfere with the DO determination have not been studied statistically.

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